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THE BIOCHEMICAL JOURNAL

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The following may be used without definition:

ADP, CDP, GDP, IDP, UDP	5′-Pyrophosphates of adenosine, cytidine, guanosine, inosine, uridine
AMP etc.	Adenosine 5′-phosphate etc.
ATP etc.	Adenosine 5′-triphosphate etc.
CM-cellulose	Carboxymethylcellulose
CoA and acyl-CoA	Coenzyme A and its acyl derivatives
DEAE-cellulose	Diethylaminoethylcellulose
DNA	Deoxyribonucleic acid
DNP-	2,4-Dinitrophenyl-
EDTA	Ethylenediaminetetra-acetate
FAD	Flavine–adenine dinucleotide
FMN	Flavine mononucleotide
GSH, GSSG	Glutathione, reduced and oxidized
NAD	Nicotinamide–adenine dinucleotide
NADP	Nicotinamide–adenine dinucleotide phosphate
NMN	Nicotinamide mononucleotide
RNA	Ribonucleic acid
P_i, PP	Orthophosphate, pyrophosphate
tris	2-Amino-2-hydroxymethylpropane-1,3-diol

Abbreviations for amino acids and sugars (see *Instructions to Authors*) are for use only in representing polymers or sequences and in Tables and Figures, and need not be defined.

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CONVERSION OF [^{14}C]ORSELLINIC ACID AND [^{14}C]ORCINOL INTO FUMIGATOL BY *ASPERGILLUS FUMIGATUS* I.M.I. 89353

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(*Received* 9 *July* 1965)

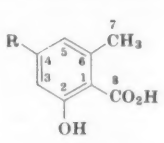
1. Sodium [1-^{14}C]acetate was incorporated into orsellinic acid and fumigatol by *Aspergillus fumigatus*. 2. [^{14}C]Orsellinic acid was prepared biosynthetically. It was converted almost entirely into fumigatol and fumigatin within 2 days of supplementation of the medium. The apparent decrease in incorporation after a longer period of growth was due to decomposition of radioactive fumigatol and the production of relatively unlabelled material. The addition of orcinol to these cultures decreased the conversion of [^{14}C]orsellinic acid into fumigatol. [^{14}C]-Orsellinic acid was incorporated into 3,4-dihydroxytoluquinol in both sets of cultures. 3. [^{14}C]Orcinol was prepared from [^{14}C]orsellinic acid after acid hydrolysis. It was also very effective as a precursor of fumigatol (60% incorporation). 4. The specific activity of fumigatin was lower than that of fumigatol at early stages of growth (4–5 days after inoculation) with all the labelled substrates that were tested. This indicated that fumigatin arose from fumigatol after oxidation in the medium. 5. The presence of orcinol in the medium greatly stimulated the incorporation of radioactivity (presumably derived from the $^{14}CO_2H$ of orsellinic acid) into the isoprenoid compounds, ergosterol and ubiquinone, in the mycelium.

Many phenolic compounds are synthesized by fungi from one molecule of acetyl-CoA plus a number of malonyl-CoA units, e.g. 6-methylsalicylic acid (I) (Bu'Lock, Smalley & Smith, 1962), orsellinic acid (II) (Mosbach, 1961), javanicin, a dihydroxynaphthaquinone derivative (Gatenbeck & Bentley, 1965), and islandicin (1,4,5-trihydroxy-2-methylanthraquinone) (Gatenbeck, 1962). Some of these substances are secreted into the culture medium. Fumigatol (III) and its oxidation product, fumigatin, are synthesized in excellent yield from [^{14}C]acetate by *Aspergillus fumigatus* (Packter & Glover, 1965*a*) and are presumably also formed via the 'acetate-plus-malonate' pathway.

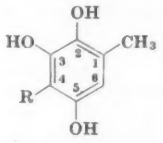
It has been suggested that the phenolic acids, 6-methylsalicylic acid and orsellinic acid, may be intermediates in the biosynthesis of other aromatic compounds in fungi (Birch, 1961; Bentley, 1962). Indeed, it has recently been shown that 6-methylsalicylic acid is converted into fumigatol and fumigatin in *A. fumigatus* (Packter, 1965). It can also be hydroxylated at C-4 to form orsellinic acid, a normal metabolite in this organism. The results described by Packter (1965) indicated that 6-methylsalicylic acid was initially converted into another substance before it could be incorporated into fumigatol and fumigatin, and it seemed probable that orsellinic acid was the true precursor. As orsellinic acid and fumigatol are both synthesized from C_2 units derived from acetate, it was decided to determine the specific activities of these metabolites with sodium [1-^{14}C]acetate as labelled substrate, at various stages of growth. In addition, [^{14}C]orsellinic acid was prepared biosynthetically and was tested directly for precursor activity of fumigatol and fumigatin in *A. fumigatus*. [^{14}C]-Orcinol was formed by decarboxylation of [^{14}C]-orsellinic acid and was similarly tested.

EXPERIMENTAL

Organisms. The fungi used during this work were obtained from the Commonwealth Mycological Institute (Kew, Surrey) and were: *A. fumigatus* (I.M.I. 89353) and *Penicillium barnense* (I.M.I. 40590). They were cultured as described previously (Packter, 1965).



(I) R=H
(II) R=OH



(III) R=O·CH$_3$
(IV) R=OH

Isolation of metabolites. Orsellinic acid, fumigatin and fumigatol were isolated from *A. fumigatus* and resolved from each other by column chromatography on silicic acid–Celite (2:1, w/w). They were estimated spectrophotometrically by using their *E* values at $\lambda_{max.}$ 300, 263 and 285 mμ respectively. Orsellinic acid was further purified on thin-layer plates of Kieselgel G (E. Merck A.-G., Darmstadt, Germany) and finally crystallized from chloroform after dilution with carrier material. Fumigatin was rechromatographed, crystallized from cyclohexane and acetylated. Fumigatol was rechromatographed, oxidized to fumigatin and in most instances converted into the acetyl derivative (Packter, 1965). Orcinol, if present, would be eluted with light petroleum–ether (3:2, v/v) and resolved from fumigatin after removal of the latter with cyclohexane. It is stable in autoclaved medium for at least 48 hr.

Another hydroxyquinone (referred to as compound 'Q') and the corresponding quinol have been isolated from certain cultures of *A. fumigatus* incubated with ^{14}C-labelled 6-methylsalicylic acid (Packter, 1965) or [^{14}C]-orsellinic acid (Table 3). The structure of this substance had not been fully established but its properties are very similar to those of fumigatin. It therefore seemed probable that it was 3,4-dihydroxytoluquinone. Accordingly, this quinone was synthesized by treating fumigatol (100 mg.) with 2 ml. of HI and red phosphorus at 100° for 15 min. The reaction products were extracted with ether. The resulting extract was chromatographed and the quinones were eluted with light petroleum–ether (11:9, v/v). Fumigatin was removed by solution in cyclohexane and the residual material was crystallized from cyclohexane–benzene (1:1, v/v) to give purple clusters of 3,4-dihydroxytoluquinone (5% yield). This substance was identical with compound 'Q' as evidenced by colour reactions with (*a*) NaOH and (*b*) ethyl cyanoacetate, and by their respective ultraviolet- and infrared-absorption spectra (Packter, 1965).

Unsaponifiable lipid, ergosterol and ubiquinone were also isolated as described previously (Packter & Glover, 1965*a*; Packter, 1965). Ergosterol was estimated spectrophotometrically by using its *E* value at 282 mμ (Mercer & Glover, 1961).

All substances were purified to constant specific activity.

[^{14}C]Orsellinic acid was prepared biosynthetically from sodium [1-^{14}C]acetate (The Radiochemical Centre, Amersham, Bucks.). This was added to the culture medium of *P. barnense* after 7 days' growth and incubation was continued for a further 7 days. [^{14}C]Orsellinic acid was isolated and purified by crystallization to give anhydrous crystals, m.p. 183–184° and specific activity 4030 counts/min./μmole. Some of this material was diluted with carrier and the total was then crystallized from chloroform. [^{14}C]Orcinol was obtained from this material (specific activity 1505 counts/min./μmole) after reaction with N-HCl for 30 min. The $^{14}CO_2$ was passed into 10 ml. of N-NaOH and counted as $BaCO_3$. The residual solution was diluted with water and extracted with ether to give a white solid with an ultraviolet spectrum identical with that of orcinol. Inflexions at 262 or 300 mμ were absent, indicating that no orsellinic acid remained. The [^{14}C]orcinol was purified by crystallization from benzene and the anhydrous crystals had m.p. 107° and specific activity 1220 counts/min./μmole. These results confirm the findings of Birkinshaw & Gowlland (1962) and Mosbach (1960) that [^{14}C]orsellinic acid obtained in this way was equally labelled in C-2, C-4, C-6 and the carboxyl group (C-8) (Table 1).

Spectrophotometry. Ultraviolet-absorption measurements were carried out in cyclohexane ('special for spectroscopy'; British Drug Houses Ltd., Poole, Dorset) or ethanol (refluxed over KOH and Zn, and then redistilled) in a Hilger and Watts Ultrascan.

Assay of radioactivity. Radioactivity was measured by counting portions of the various fractions on planchets in

Table 1. *Decarboxylation of orsellinic acid*

Experimental details are given in the text. The numbers of the labelled carbon atoms are given in parentheses.

	Specific activity	
	(counts/min./μmole)	(counts/min./μg.atom of ^{14}C)
Orsellinic acid (C-2, C-4, C-6 and C-8)	1505	376
Orcinol (C-2, C-4 and C-6)	1220	407
$BaCO_3$ (C-8)	355	355

Table 2. *Incorporation of sodium* [1-^{14}C]*acetate into orsellinic acid, fumigatol and fumigatin*

Sodium [1-^{14}C]acetate (5 μc; specific activity 2·9 μc/μmole) was added to each of nine Roux bottles containing 200 ml. of Raulin–Thom medium after 3 days' growth; three bottles were subsequently removed at intervals. All values are expressed as totals/600 ml. of medium. The specific activity of orsellinic acid has been corrected for the addition of carrier material.

Group	Total incubation time (days)	pH of medium	Ether extract (mg.)	Total activity (counts/min.)	Orsellinic acid (mg.)	Specific activity (counts/min./μmole)	Fumigatol (mg.)	Specific activity (counts/min./μmole)	Fumigatin (mg.)	Specific activity (counts/min./μmole)	Total activity in fumigatol and fumigatin (counts/min.)
1	4	2·8	108	213000	0·3	1025	65	306	2·1	276	122000
2	5	2·8	170	181000	0·5	109	105	255	4·7	252	157500
3	6	3·0	258	143000	0·5	55	131	151	4·0	153*	125490

* The specific activity of 3,4-dihydroxytoluquinone was 94 counts/min./μmole.

a Nuclear–Chicago Corp. gas-flow counter (25% efficiency). Equal amounts (usually 0·2ml.) were estimated spectrophotometrically. Samples were counted at infinite thinness and corrected for background. All specific activities are expressed as counts/min./μmole.

Melting points. These were determined in unsealed capillary tubes in a Gallenkamp melting-point apparatus and are uncorrected.

RESULTS

Incorporation of sodium [1-^{14}C]*acetate into orsellinic acid and fumigatol.* The values for the specific activities of orsellinic acid, fumigatol and fumigatin at different time-intervals are given in Table 2. The [^{14}C]acetate was rapidly incorporated into these metabolites. The specific activity of orsellinic acid isolated from group 1 was considerably higher than that of fumigatol. At later stages of growth, the specific activity of orsellinic acid fell to about one-twentieth of this value. Most of the activity present in the ether-extractable material of groups 2 and 3 was concentrated in the fumigatol and fumigatin fractions (approx. 90%). The specific activity of fumigatol was somewhat higher than that of the quinone in group 1 but they were identical in groups 2 and 3. A small amount of 3,4-dihydroxytoluquinone (0·8mg.) was isolated from the medium of group 3; its specific activity was lower than that of fumigatol.

These results were all reproduced in a confirmatory experiment.

Conversion of [^{14}C]*orsellinic acid into fumigatol.* In a preliminary experiment, 10mg. of [^{14}C]orsellinic acid was added to each of three Roux bottles after 3 days' growth and incubation proceeded for a further 3 days. Fumigatol and fumigatin were isolated and both were highly labelled; their combined radioactivities accounted for over 80% of that found in the ether-extractable material. The incorporation of label into these metabolites was 80% (after correction for loss of the $^{14}CO_2H$ group). 3,4-Dihydroxytoluquinone was also present and its specific activity was lower than that of fumigatin.

Next, the incorporation of [^{14}C]orsellinic acid into fumigatol and fumigatin was measured after 5 and 8 days of incubation. Further, as the first stage in the conversion of orsellinic acid into fumigatol might be decarboxylation to orcinol, the possible dilution effect of this substance on incorporation was also tested. Details of the metabolites isolated from the medium are shown in Table 3. Considering groups 1 and 2, which were harvested 2 days after supplementation of the medium, both fumigatol and fumigatin were highly active. The specific activity of fumigatin, however, was lower than that of fumigatol. Orsellinic acid was isolated from these groups and its specific activity in both cases had been markedly decreased from its initial value.

Table 3. *Effect of orcinol on the incorporation of* [^{14}C]*orsellinic acid into fumigatol*

Orcinol (25mg./200ml.) was added to each of three Roux bottles in groups 2 and 4 respectively after 3 days' growth. [^{14}C]Orsellinic acid (7mg.; specific activity 4030 counts/min./μmole) was added to each bottle 4hr. later. Incubation proceeded for a further 2 days (groups 1 and 2) or 5 days (groups 3 and 4). All values are expressed as totals/600ml. of medium. The specific activity of the orsellinic acid isolated from the medium has been corrected for the addition of carrier material. The incorporation values have been determined on a basis of 1 μg.-atom of ^{14}C. The pH of the medium of groups 1, 2, 3 and 4 was 2·2, 2·2, 4·2 and 4·8 respectively.

Group	Ether extract (mg.)	Total activity of ether extract (counts/min.)	Total activity of aqueous residue (counts/min.)	Orsellinic acid (mg.)	Specific activity (counts/min./μmole)	Fumigatol (mg.)	Specific activity (counts/min./μmole)	Fumigatin (mg.)	Specific activity (counts/min./μmole)	Total activity in fumigatol and fumigatin (counts/min.)	Incorporation of orsellinic acid into fumigatol and fumigatin (%)	3,4-Dihydroxytoluquinol (mg.)	Specific activity (counts/min./μmole)
1	258	372900	—	0·7	336	134	450	3·9	393	363560	95	7	292
2	320	319300	—	0·8	220	156	312	3·2	202	290290	77	6	241
3	245	132900	155300	0·2	—	97	185	3·5	181	109720	29	4	56
4	302	126360	98500	0·2	—	95	133	3·0	138	74350	19	2	58

The specific activity of 3,4-dihydroxytoluquinol was lower than that found for fumigatol. In estimating the amount of this substance present, it was assumed that approx. 15% had been converted into the quinone (i.e. similar to the yield obtained in the oxidation of fumigatol). Over 90% of the activity in the ether-extractable material was present in the fumigatol and fumigatin fractions.

A different pattern of results was obtained in groups 3 and 4. The total activity present in the ether-extractable matter was much less in these groups and this was confirmed by the lower specific activities of fumigatol and fumigatin. Moreover, the aqueous medium possessed considerable activity. Insufficient orsellinic acid was detected to permit purification.

The specific activities of fumigatol and fumigatin isolated from the orcinol-supplemented groups were lower than the corresponding values in groups 1 and 3 (Table 3). The synthesis of 3,4-dihydroxytoluquinol was not affected.

Determination of radioactivity in some unsaponifiable lipids in the mycelium. Most of the radioactivity present in the unsaponifiable lipid fraction was due to ergosterol (Table 4). Ubiquinone was also labelled and possessed about the same specific activity as ergosterol. The residual radioactivity in the unsaponifiable matter was presumably due to the presence of other unidentified sterols.

The incorporation of radioactivity into isoprenoid-derived compounds was greatly stimulated by orcinol (groups 2 and 4).

Conversion of [^{14}C]*orcinol into fumigatol.* The results obtained in this experiment are given in Table 5 and were similar to those determined for [^{14}C]orsellinic acid. [^{14}C]Orcinol was very effec-

Table 4. *Incorporation of activity from* [^{14}C]*orsellinic acid into some unsaponifiable lipids in the mycelium*

Experimental details are given in Table 3. The specific activity of ubiquinone has been corrected for the addition of carrier material.

	Unsaponifiable lipid		Ergosterol				Ubiquinone		
					Specific activity			Specific activity	
Group	Weight (mg.)	Total activity (counts/min.)	Weight (mg.)	Total activity (counts/min.)	(counts/min./μmole)	(counts/min./μg.atom of ^{14}C)	Weight (mg.)	(counts/min./μmole)	(counts/min./μg.atom of ^{14}C)
1	123	235	15·1	151	4	0·7	0·69	0	0
2	220	4690	9·9	695	24	4·0	0·61	20	2·0
3	252	950	48·1	624	5	0·8	0·80	4	0·4
4	265	6950	40·4	4440	44	7·3	0·92	77	7·7

Table 5. *Conversion of* [^{14}C]*orcinol into fumigatol*

[^{14}C]Orcinol (5 mg.; specific activity 1220 counts/min./μmole) was added to each of six Roux bottles after 2·5 days' growth. Incubation proceeded for a further 2 and 4·5 days (groups 1 and 2 respectively); each group consisted of three Roux bottles and the results are expressed as totals/600 ml. of medium. The pH of the medium of groups 1 and 2 was 2·3 and 2·4 respectively.

	Group 1			Group 2		
	Weight (mg.)	Total activity (counts/min.)	Specific activity (counts/min./μmole)	Weight (mg.)	Total activity (counts/min.)	Specific activity (counts/min./μmole)
Medium						
Ether extract	229	50620	—	300	22500	—
Aqueous residue	—	< 5000	—	—	24100	—
Fumigatol	103	43570	71	130	14560	20
Fumigatin	3·6	1190	55	4·4	506	19
Orsellinic acid	0·7	0	0	0·5	0	0
Mycelium						
Unsaponifiable lipid	90	900	—	139	790	—
Ergosterol	18	0	0	29	0	0

tively converted into fumigatol and fumigatin (57 and 1·5% respectively) within 2 days of supplementation of the medium (group 1). The specific activity of fumigatin was again lower than fumigatol at this stage of growth (compare group 1 in Table 2 and groups 1 and 2 in Table 3). Nearly 90% of the radioactivity in the ether-extractable material was located in the fumigatol and fumigatin fractions. Orsellinic acid was isolated but, as expected, possessed no radioactivity. Orcinol was not present in significant amounts (less than 0·1 mg.). Similar results were obtained for group 2. This group, however, contained less radioactivity than the first, but the aqueous medium after ether extraction was appreciably radioactive.

Trace amounts of 3,4-dihydroxytoluquinone (0·4 mg.) were detected in group 1.

The unsaponifiable lipid in groups 1 and 2 did not possess appreciable radioactivity. Ergosterol was not radioactive.

DISCUSSION

The results described in Table 2 are consistent with the view that orsellinic acid is a normal intermediate in the biosynthesis of fumigatol from acetyl-CoA. In group 1, the specific activity of orsellinic acid was much higher than that of fumigatol, but in the other groups at later stages of growth it fell below that value. However, at this time, the pool of [^{14}C]acetyl-CoA would have been greatly diluted by endogenously produced material and substances subsequently formed from acetyl-CoA would have a lower specific activity. Accordingly, as only 0·3 mg. of orsellinic acid was present in the medium after 4 days' growth, the secretion of a further milligram, for example, of unlabelled substance during the fifth and sixth day of incubation would decrease the specific activity considerably. It is evident from the comparative amounts and specific activities of orsellinic acid that the mycelium is freely permeable to this substance. This is confirmed by the results given in Table 3. By contrast, the situation with fumigatol is quite different. The cultures in group 1 had already secreted an appreciable amount of fumigatol into the medium, and non-radioactive material synthesized later would not affect the specific activity to the same extent. Presumably, fumigatol does not re-enter the mycelium.

[^{14}C]Orsellinic acid is converted almost entirely into fumigatol within 2 days (Table 3). It is also incorporated into 3,4-dihydroxytoluquinol (IV), but on all occasions the specific activity of this substance was appreciably lower than that of fumigatol. The addition of orcinol to the medium effectively diluted out the incorporation of orsellinic acid into fumigatol. The synthesis of 3,4-dihydroxytoluquinol was not affected. This is in marked contrast with the effect of orcinol on the conversion of ^{14}C-labelled 6-methylsalicylic acid into fumigatin and 3,4-dihydroxytoluquinone (Packter, 1965). The incorporation into fumigatin was decreased to nearly zero and at the same time the cultures were stimulated to synthesize 3,4-dihydroxytoluquinone, with a very high specific activity. Significant quantities of 3,4-dihydroxytoluquinol or the corresponding quinone are only present when the medium has been supplemented with orsellinic acid or 6-methylsalicylic acid together with orcinol. These results suggest that 3,4-dihydroxytoluquinol is not an intermediate in the biosynthesis of fumigatol.

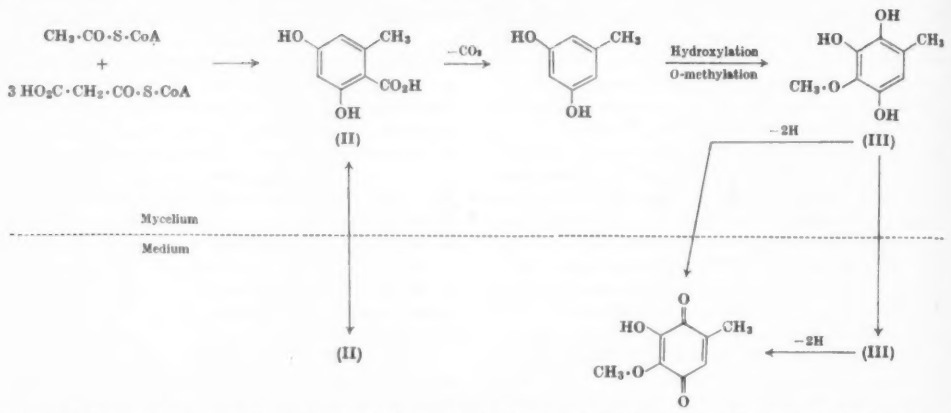
The apparently diminished incorporation into fumigatol and 3,4-dihydroxytoluquinol over longer periods of incubation may be explained by the fact that these metabolites decompose in aqueous solution into ether-insoluble compounds. Indeed, the residual medium, after extraction with ether, possessed a considerable amount of radioactivity (Tables 3 and 5). Presumably this was due mainly to the presence of breakdown products of fumigatol.

[^{14}C]Orcinol is also very effectively and quickly converted into fumigatol (60% incorporation). Ergosterol, isolated from the mycelium, is unlabelled, and so presumably none of the [^{14}C]orcinol has been metabolized to [^{14}C]acetyl-CoA.

The results therefore indicate that orsellinic acid is formed from acetyl-CoA and then decarboxylated. The orcinol thus produced may be hydroxylated and *O*-methylated to form fumigatol. However, the results expressed in Tables 3 and 5 do not rule out the possibility that orsellinic acid may be hydroxylated initially to produce a trihydroxy acid, for example, which could be the true precursor. Orcinol has not been detected in the culture medium of *A. fumigatus* but it has been found, together with orsellinic acid, 5-methylorsellinic acid and 6-methylsalicylic acid, in the medium of mutants of *Aspergillus terreus* and in shake-cultures of the wild type (Curtis, Harries, Hassall & Levi, 1964). Further, it has been established that orsellinic acid is decarboxylated by *Gliocladium roseum* (M. W. Steward & N. M. Packter, unpublished work).

A previous report has already suggested that [^{14}C]orsellinic acid might be incorporated into fumigatin in *A. fumigatus* (Pettersson, 1963). However, the fumigatin in this work was not obtained in a crystalline state. The medium was oxidized before extraction and fumigatol was not isolated. Further, the spectral properties quoted for fumigatin do not agree with those established by Packter & Glover (1962) for the pure crystalline material.

The specific activity of fumigatol was higher than that of the quinone in group 1 of the [^{14}C]acetate

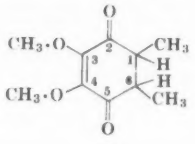


Scheme 1. Proposed sequence of reactions for the biosynthesis of fumigatol and formation of fumigatin by *A. fumigatus*.

experiment but their activities were equal in groups 2 and 3 (Table 2). This latter result is in agreement with that reported by Packter & Glover (1965*a*) for cultures of *A. fumigatus* incubated with [^{14}C]-acetate for 3 days. The total incubation time was 7 days. In the experiment described in Table 2, the medium was pale orange when the [^{14}C]acetate was added; some fumigatin (and presumably fumigatol) therefore was present already. Accordingly, the specific activity of the small amounts of ^{14}C-labelled fumigatin produced by oxidation of fumigatol would initially be lowered. This effect would become less marked subsequently. A similar pattern of results was also obtained when [^{14}C]orsellinic acid or [^{14}C]orcinol was the labelled substrate. Packter (1965) has indicated that most of the fumigatin present in the medium could arise from oxidation of fumigatol. Some fumigatin, however, is secreted as such after 7 days' incubation.

Thus the results obtained during this work support the proposed pathway of fumigatol biosynthesis and fumigatin formation that is outlined in Scheme 1. No evidence is available as to which carbon atom of orcinol is hydroxylated first. The reported lack of incorporation of 4[^{14}C]-methoxytoluquinone into fumigatol (Packter & Glover, 1965*b*) is readily explained as a hydroxyl group at C-3 of the toluquinol ring would appear to be necessary for precursor activity.

Orsellinic acid might also act as a precursor of gliorosein (Brian, Curtis, Howland, Jefferys & Raudnitz, 1951) in *G. roseum*. Gliorosein (1,6-dihydro form, established by its n.m.r. spectrum) (V) is slowly isomerized in the medium during incubation into the corresponding *p*-quinol, which is itself oxidized to form rubrogliocladin (quinhydrone) and aurantiogliocladin (quinone) (Steward



(V)

& Packter, 1965). A preliminary report by Birch (1961) has indicated that [5-^{14}C]orsellinic acid (prepared synthetically) is not incorporated into aurantiogliocladin. No experimental details were given. Recent studies in this Laboratory (M. W. Steward & N. M. Packter, unpublished work) have shown that [^{14}C]orsellinic acid is rapidly decarboxylated by *G. roseum* and an approximately equivalent amount of orcinol is secreted into the medium. This suggested that orcinol does not act as a precursor either. It is possible, however, that orsellinic acid and orcinol are involved in the biosynthesis of gliorosein *in vivo* and react as enzyme complexes. The formation of enzyme-bound intermediates in acetyl-CoA and malonyl-CoA condensation reactions has now been well established in fatty acid (Wakil, Pugh & Sauer, 1964) and mevalonic acid biosynthesis (Brodie, Wasson & Porter, 1964).

During the conversion of [^{14}C]orsellinic acid into fumigatol, the carboxyl group is eliminated as $^{14}CO_2$. At the same time, radioactivity is incorporated into the isoprenoid-derived compounds, ergosterol and ubiquinone, in the mycelium. Goodwin (1959) has demonstrated that $^{14}CO_2$ is fixed into β-carotene by *Phycomyces blakesleeanus* when leucine is present in the medium. Further, Packter & Glover (1962, 1965*b*) have established that small amounts of L-leucine (0·1–0·2g./200ml.)

stimulate the synthesis of ubiquinone in *A. fumigatus*. These observations are readily explained by the work of Coon, Kupiecki, Dekker, Schlesinger & del Campillo (1959), who indicated that β-methylcrotonyl-CoA, a product of leucine catabolism, may be carboxylated to give β-methylglutaconyl-CoA and β-hydroxy-β-methylglutaryl-CoA, a precursor of isoprenoid compounds. Ergosterol does not become labelled when ^{14}C-labelled 6-methylsalicylic acid is used as substrate (Packter, 1965). However, in this case only 5% is decarboxylated to *m*-cresol and 1–1·5% is converted into fumigatin and fumigatol. Further, these reactions do not occur until a late stage of growth. Accordingly, only a small amount of $^{14}CO_2$ is available for fixation into β-methylglutaconyl-CoA at a time when there would be little turnover of isoprenoid compounds.

No explanation can be given, as yet, for the apparent stimulation of incorporation of radioactivity into ergosterol and ubiquinone (and presumably other products of terpene biosynthesis) in the presence of orcinol.

I thank Mrs D. McIlreavy and Mr D. Beaumont for their very able technical assistance at different stages of this work. Thanks are also due to Mr M. Thomas, who performed part of the [^{14}C]acetate experiment.

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The Phosphorylated Intermediate in the Phosphoglyceromutase Reaction

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(*Received* 6 *August* 1965)

1. High-voltage paper-electrophoresis methods have been used for the separation of ^{32}P-labelled phosphoesters. 2. Evidence is presented which indicates that ^{32}P-labelled phosphopeptides, obtained after acid hydrolysis of phosphoglyceromutase incubated with impure 2,3-di[^{32}P]phosphoglycerate, are derived from phosphoglucomutase contamination. 3. The hydrolysis of 2,3-di[^{32}P]phosphoglycerate by phosphoglyceromutase has been studied. After an apparent instantaneous hydrolysis of 1 mole of coenzyme/mole of enzyme the reaction proceeds at a very low rate. 4. This hydrolysis seems to be due to the destruction of an enzyme–coenzyme complex. The proportions of the decomposition products of the complex vary according to further handling (pH of ionophoresis). 5. The inorganic [^{32}P]-phosphate produced by the hydrolysis of the complex and the inorganic [^{32}P]phosphate produced by the slow phosphatase activity can be differentiated by the ability of the former to be incorporated into non-radioactive substrate before enzyme denaturation. 6. The effect of enzyme concentration on the stoicheiometry of the slow phosphatase hydrolysis of the diphosphoglycerate is described and this suggests that it may occur via two independent reactions, one of them being the decomposition of the enzyme–coenzyme complex on standing.

Phosphoglyceromutase (2,3-diphospho-D-glycerate–2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3) catalyses the intramolecular transfer of the phosphate of 2- and 3-phosphoglycerate. Since the discovery by Sutherland, Posternak & Cori (1949) that a diphosphate cofactor (identified as 2,3-diphosphoglycerate) is involved in the reaction, its mechanism has been associated with the mechanism of the phosphoglucomutase (EC 2.7.5.1) reaction. In the latter a phosphoenzyme has been postulated which is able to phosphorylate either glucose 1-phosphate or glucose 6-phosphate to give the cofactor glucose 1,6-diphosphate (Najjar & Pullman, 1954). That phosphate is covalently bound to this enzyme was shown by Anderson & Jolles (1957), who isolated phosphoserine from acid hydrolysates; the amino acid sequence around this particular phosphoserine was shown to be (Milstein & Sanger, 1961) Thr-Ala-phosphoserine-His-[Asp or Asp(NH_2)]. A similar mechanism was proposed by Pizer (1960) for the phosphoglyceromutase reaction based on the finding that labelled phosphoserine was obtained from a partially acid-hydrolysed phosphoglyceromutase preparation equilibrated with [^{32}P]diphosphoglycerate.

More recently, Grisolia, Joyce & Fernandez (1961) reported that a fraction of the phosphoprotein produced after incubation with the diphosphoglycerate did not equilibrate with the substrate. The possibility that phosphoserine arose from a contamination with phosphoglucomutase was mentioned (Pizer, 1962). Acid-labile phosphate was shown to remain associated with the muscle enzyme, after incubation with diphosphoglycerate and purification on a basic resin, in the ratio of 2 phosphate residues/mole of protein. This bound phosphate was shown to equilibrate with substrate (Grisolia *et al.* 1961).

Experimental evidence indicating that the acid-stable phosphorylated enzyme is a phosphoglucomutase contaminant and that the acid-labile phosphorylated protein is an intermediate enzyme–coenzyme complex are presented in this paper. The experimental approach used to disclose this complex may be of more general use for obtaining evidence on the existence of rather unstable intermediates. The complex decomposes under mild conditions giving inorganic phosphate and monophosphoglycerate in different proportions depending on the conditions. The 2,3-diphosphoglycerate-phosphatase activity of the enzyme has been reinvestigated. A preliminary report on part of this work has been published (Zwaig & Milstein, 1963).

MATERIAL AND METHODS

[^{32}P]*Phosphoesters.* A crude mixture of ^{32}P-labelled phosphoesters was prepared by two methods.

Method 1. ^{32}P was incorporated into the phosphoesters by using erythrocytes (Prankerd & Altman, 1954). The erythrocytes from 1ml. of rabbit blood were incubated with 1mc of ^{32}P (inorganic phosphate) as indicated by Grisolia *et al.* (1961) and were then centrifuged and washed twice with 3ml. of 0·9% NaCl. The erythrocytes were haemolysed with 5ml. of water and proteins were precipitated in a boiling-water bath for 30min. The supernatant was concentrated in an evacuated desiccator containing H_2SO_4.

Method 2. In this method purified or partially purified enzymes were used to incorporate the ^{32}P into the phosphoesters. This gave maximum specific radioactivity and was used at early stages of the work. However, the yield of radioactive esters was rather low and not reproducible, varying with the batch of radioactive phosphate. For this reason method 1 was preferred.

The incubation mixture contained: 15μmoles of fructose diphosphate, 15μmoles of $MgSO_4$, 30mμmoles of K_2HPO_4 containing 1mc of ^{32}P, 0·2mg. of NAD, 50μmoles of tris, pH8·0, 0·5μmole of sodium pyruvate, 40μg. of crystalline aldolase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany), 25μg. of crystalline glycerophosphate dehydrogenase (a kind gift of Dr J. Park) and 15μl. of a water extract of chicken breast (Rodwell, Towne & Grisolia, 1957). The final volume was 1ml. The mixture was incubated for 1hr. at 38° and the reaction was then stopped with a few drops of acetic acid. Up to 7% of ^{32}P was converted into 2,3-diphosphoglycerate.

Purification of 2,3-*di*[^{32}P]*phosphoglycerate.* The crude mixture of ^{32}P-labelled phosphoesters prepared by either method 1 or method 2 was subjected to paper electrophoresis at pH3·5 with Whatman 3MM paper in a 20cm.-wide band. The position of the esters was located by using suitable markers and the diphosphoglycerate, free from radioactive contaminants, was eluted with distilled water. At most pH values the diphosphoglycerate was well separated from other phosphoesters and also from inorganic phosphate (Fig. 1). This purification procedure was considered better than paper chromatography in propanol–NH_3–water as used by Pizer (1960) since, in the latter, glucose 1,6-diphosphate and 2,3-diphosphoglycerate are not separated.

Labelling of phosphoglyceromutase. Labelling of the enzyme was attempted by several procedures with labelled diphosphoglycerate or a mixture of labelled phosphoesters. Two methods are described in detail.

Method (*a*). A crude mixture of ^{32}P-labelled phosphoesters was used in this method. A portion (2·5ml.) of the haemolysed erythrocytes containing ^{32}P (prepared as indicated in method 1 above) was placed in a 10ml. conical tube and allowed to equilibrate for 48hr. at 4° with 2·5mg. of phosphoglyceromutase, which was dissolved in a minimum volume of water and placed in a small dialysis bag. The enzyme was then dialysed with stirring for 24hr. against distilled water, which was changed at 30min. intervals by a siphoning procedure. The dialysis bag was fixed at one end and a trapped air bubble ensured good mixing of its content during the process of siphoning and refilling of the outside container.

Method (*b*). [^{32}P]Diphosphoglycerate purified by ionophoresis at pH6·5 was used in this method. Phosphoglyceromutase (2mg. dissolved in 0·033M-tris, pH7·0) was incubated with about 0·2μmole of 2,3-di[^{32}P]phosphoglycerate, containing about $7{\cdot}5\times10^5$ counts/min. The total volume was 0·6ml. The incubation was carried out for 3hr. at 31°. The preparation was then dialysed until negligible radioactivity was detected in the diffusate.

Partial acid hydrolysis. This was carried out at 100° with 5·6N-HCl for 30min. (Milstein & Sanger, 1961).

Trypsin digestion. ^{32}P-labelled phosphoglyceromutase (1mg.; method *b*) was denatured by incubation for 10min. in a boiling-water bath. Ammonium hydrogen carbonate was added to give a final concentration of 0·5% and 10μg. of trypsin (crystalline; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was added. Digestion was carried out at 37° for 20hr. The material was dried *in vacuo* over NaOH. Residual salt was removed by two successive additions of water, the material being dried after each addition. The products were subjected to ionophoresis and radioautography (Milstein, 1964). A mixture of dyes was used as visual indication of the run (Milstein & Sanger, 1961).

Enzyme activity. Phosphoglyceromutase activity was estimated by the method of Sutherland *et al.* (1949). Phosphoglucomutase activity was measured as described by Milstein (1961).

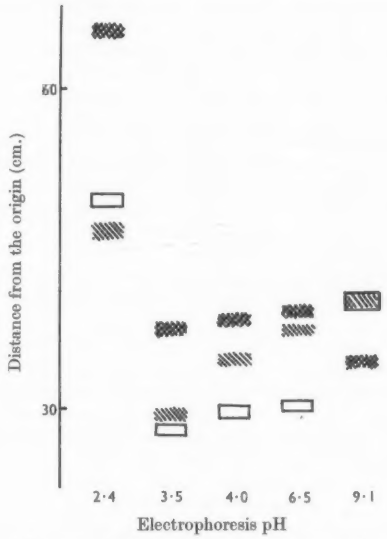


Fig. 1. Diagram showing the separation of monophosphoglycerate from 2,3-diphosphoglycerate and inorganic phosphate by paper electrophoresis. ▭, Inorganic phosphate; ▨, 2- and 3-phosphoglycerate; ▩, 2,3-diphosphoglycerate.

pH2·4: formic acid–pyridine–water (3:0·5:96·5, by vol.);
pH3·5: pyridine–acetic acid–water (1:10:189, by vol.);
pH4·0: from pH3·5, by adding pyridine;
pH6·5: pyridine–acetic acid–water (10:0·4:90, by vol.);
pH9·1: ammonium carbonate–water (1:9, w/w).

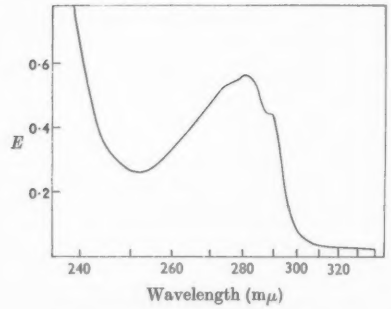


Fig. 2. Ultraviolet spectrum of phosphoglyceromutase (1·344 mg. dry wt. dissolved in 3·00 ml. of water in a 1 cm.-path cell). The spectrum was determined in a Beckmann DK-2A automatic recorder spectrophotometer.

Inorganic phosphate. This was estimated by the method of Fiske & Subbarow (1925) or by the method of Bartlett (1959) when only very small quantities were available.

Protein concentration. This was estimated from the extinction at 280 mμ. The u.v. spectrum of the protein is shown in Fig. 2. The extinction coefficient of crystalline phosphoglyceromutase was determined on a dry-weight basis: $E^{1\%}_{280\,m\mu}$ 12·5.

Other methods. Paper electrophoresis, radioautography and radioactive measurements were carried out as described by Milstein (1964).

Phosphoglyceromutase. This enzyme, from rabbit muscle, was obtained as a crystalline suspension in 2·6 M-$(NH_4)_2SO_4$ from C. F. Boehringer und Soehne G.m.b.H. and dialysed against glass-distilled water before use. ^{32}P-labelled phosphoglucomutase was prepared according to Milstein & Sanger (1961). Glucose 1,6-diphosphate was a kind gift of Dr C. Cardini and 2,3-diphosphoglycerate was kindly provided by Dr S. Grisolia.

RESULTS

Binding of ^{32}P *to phosphoglyceromutase*

Preliminary experiments to study the binding of ^{32}P to phosphoglyceromutase were made with crude muscle extracts, equilibrated with a mixture of ^{32}P-labelled phosphoesters by the dialysis technique employed to label phosphoglucomutase (Milstein & Sanger, 1961). Separation of phosphoglyceromutase and phosphoglucomutase activities can be achieved on a DEAE-cellulose column at pH 8·3 and a gradient of tris from 0·02 to 0·2 M. Phosphoglucomutase is retained in the column and phosphoglyceromutase comes straight through. These experiments did not show any radioactivity associated with the phosphoglyceromutase activity. The ionograms of radioactive peptides obtained after partial hydrolysis of the labelled protein material (which eluted together with the phosphoglucomutase activity) were the same as the one obtained from pure ^{32}P-labelled phosphoglucomutase. More thorough experiments were made with crystalline preparations of phosphoglyceromutase.

Labelling of the crystalline phosphoglyceromutase by method (*a*) (in which a mixture of ^{32}P-labelled phosphoesters was used) and by method (*b*) (in which purified 2,3-diphosphoglycerate was used) yielded different products. In method (*a*) the label was acid-stable but with method (*b*) no acid-stable phosphoprotein was obtained. An investigation of the acid-stable phosphoprotein obtained by method (*a*) was undertaken.

When the radioactive peptides obtained after partial acid hydrolysis of a ^{32}P-labelled phosphoglyceromutase preparation (method *a*) were compared by ionophoresis at pH 3·5 with those obtained from ^{32}P-labelled phosphoglucomutase, the same pattern was obtained (Fig. 3C and D). Although no precise measurements of the acid-stable ^{32}P-labelled protein were made, it became obvious that the yields were considerably lower than those obtained when ^{32}P-labelled phosphoglucomutase was prepared. Therefore the possibility that this acid-stable phosphoprotein was in fact a contaminant (and Fig. 3C and D suggested that the contaminant could be phosphoglucomutase) was considered. Trace amounts of phosphoglucomutase activity were detected in the crystalline phosphoglyceromutase preparation used. The activity present was equivalent to 0·5% (by wt.) of phosphoglucomutase as contaminant. However, the possibility of this activity being due to a lack of specificity of the phosphoglyceromutase rather than to contamination was considered.

The pattern of the ionogram of radioactive peptides obtained after partial acid hydrolysis of ^{32}P-labelled phosphoglucomutase was unchanged if 3-phosphoglycerate was incubated with the radioactive enzyme before partial acid hydrolysis. In contrast, incubation of ^{32}P-labelled phosphoglucomutase with glucose 6-phosphate (equilibration of the radioactive phosphoenzyme with the non-radioactive substrate being allowed) gave rise to a pattern of radioactive bands in which a stronger band 3 (containing glucose phosphate; Milstein & Sanger, 1961) was obtained, and the peptide bands 4, 5 and 7 were no longer present (Fig. 3F). Band 6, on the other hand, was present after this treatment. When this band was run on ionophoresis at pH 6·5 it did not correspond to any of the peptide bands identified by Milstein & Sanger (1961) in the partial acid hydrolysate of ^{32}P-labelled phosphoglucomutase, but rather to the unidentified spot which the above authors called X.

When a similar experiment was carried out with

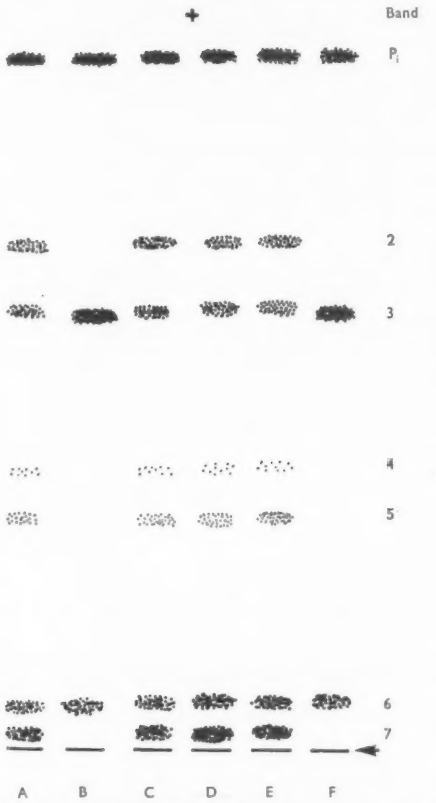


Fig. 3. Tracing of the radioautograph of the [^{32}P]peptides obtained after partial acid hydrolysis of ^{32}P-labelled phosphoglyceromutase (A, B and C) and ^{32}P-labelled phosphoglucomutase (D, E and F). ^{32}P-labelled enzymes (0·03μc) were previously equilibrated with 8μmoles of histidine, pH 7·0, 0·4μmole of $MgCl_2$ and 2μmoles of non-radioactive substrate (in 0·2ml.): A and E, 3-phosphoglycerate; B and F, glucose 6-phosphate; C and D, incubation with non-radioactive substrate omitted. Electrophoresis at pH 3·5. ^{32}P-labelled phosphoglyceromutase was prepared by method (*a*). Band numbers are as in Milstein & Sanger (1961).

the acid-stable ^{32}P-labelled phosphoglyceromutase (prepared by method *a*) the same result was obtained, namely that after incubation with glucose 6-phosphate the radioactive peptides characteristic of the sequence around the reactive serine in phosphoglucomutase were lost (Fig. 3B). Incubation with 3-phosphoglycerate on the other hand did not change the radioactive spots on the ionogram after acid hydrolysis (Fig. 3A). These results indicated that the acid-stable [^{32}P]phosphoprotein obtained by method (*a*) was indeed a contamination of phosphoglucomutase present in the crystalline phosphoglyceromutase preparation.

State of the labile ^{32}P bound to phosphoglyceromutase

When the ^{32}P-labelled phosphoglyceromutase was prepared by method (*b*) and dialysed, considerable radioactivity remained associated with the protein. But after partial acid hydrolysis or digestion with trypsin, the radioactivity was recovered as inorganic phosphate after ionophoresis at pH 3·5 and 6·5. The presence of labile bound phosphate to phosphoglyceromutase has been reported by Grisolia *et al.* (1961).

When 2,3-diphosphoglycerate was incubated with phosphoglyceromutase for long periods, a slow hydrolysis of the ester, leading to the formation of inorganic phosphate and monophosphoglycerate, was observed. The time-course of this extremely slow process is shown in Fig. 4. The formation of inorganic phosphate under these conditions was linear, but extrapolation showed that the line did not pass through the origin. Values obtained at the minimum time of 2min. indicated that a small but significant amount of diphosphoglycerate was hydrolysed. From this and other similar observations it became obvious that this amount was of the same order of magnitude as the amount (in moles) of enzyme which was being used. This apparently instantaneous hydrolysis of diphosphoglycerate was studied in considerable detail under conditions in which the phosphatase reaction was negligible. This was achieved by reducing the incubation period to a minimum (not more than 2min.) and the temperature to 0°. The incubation mixture was then immediately applied to a chromatographic paper, dried with a hair-drier and subjected to high-voltage electrophoresis. This procedure involves a loss of enzyme activity, which was shown on a control by eluting with 0·01M-tris, pH 7·0, a piece of 3MM paper containing dried enzyme and measuring the specific activity before and after application to the paper. The enzyme activity remaining after the procedure was less than 5% of the original. To obtain a reasonable accuracy, very low concentrations of diphosphoglycerate (of the same order of magnitude as the concentration of enzyme) had to be used. Therefore it was essential to work with radioactive diphosphoglycerate of very high specific activity.

When the 'burst' reaction was studied under these conditions the following results were obtained. The radioactivity of the decomposed [^{32}P]diphosphoglycerate recovered as inorganic phosphate and monophosphoglycerate depended on the pH at

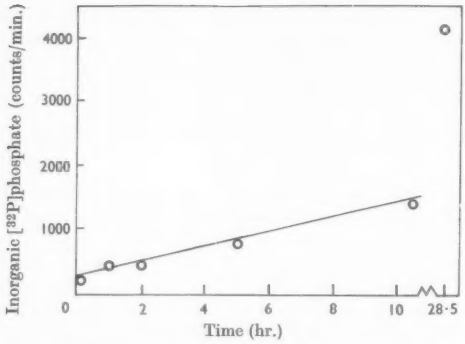


Fig. 4. Time-course of the formation of inorganic phosphate on incubation of 2,3-diphosphoglycerate with phosphoglyceromutase. 2,3-Di[^{32}P]phosphoglycerate (104 mμmoles; $2{\cdot}7 \times 10^6$ counts/min./μmole) was incubated with 1·6 mμmoles of phosphoglyceromutase in 0·3 ml. of 0·05 M-tris buffer, pH 7·0. At different times, samples were applied to a sheet of Whatman 3MM paper, immediately dried with hot air and subjected to pH 6·5 electrophoresis. The inorganic [^{32}P]phosphate was located by radioautography and the spots were counted in a gas-flow counter (Nuclear–Chicago model D–47).

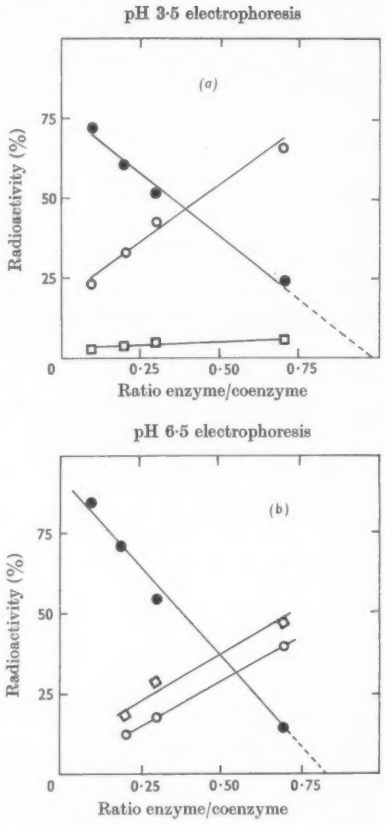


Fig. 5. Effect of enzyme/coenzyme ratio and of the ionophoretic separation procedure on the 'burst' hydrolysis of 2,3-diphosphoglycerate. 2,3-Di[^{32}P]phosphoglycerate (4·4 mμmoles; $4{\cdot}5 \times 10^6$ counts/min./μmole) was incubated for 2 min. in an ice bath with different amounts of phosphoglyceromutase in 0·27 ml. of 0·05 M-tris buffer, pH 7·0. Samples were applied on Whatman 3MM paper and dried as fast as possible with a hair-drier. Ionophoresis was carried out at pH 3·5 (Fig. 5*a*) and pH 6·5 (Fig. 5*b*). The radioactivity of the spots obtained after radioautography was measured and plotted as a percentage of the total. Radioactivity present as ●, diphosphoglycerate, □, monophosphyglycerate, ○, inorganic phosphate.

which the ionophoretic separation was carried out. Fig. 5(*a*) shows the proportion of radioactive products recovered by ionophoresis at pH 3·5 and Fig. 5(*b*) the proportion of radioactive products recovered in the same experiment by ionophoresis at pH 6·5. When the amount of coenzyme was higher than the amount of enzyme present, within the experimental error, stoicheiometric amounts of cofactor decomposed under these conditions. When the slow phosphatase activity was avoided by using very short incubations in the cold the excess of cofactor above a mole to mole ratio with enzyme was always recovered as such. When the amount of coenzyme was lower than the amount of enzyme, no coenzyme could be recovered after electrophoresis at any pH. In this experiment it was observed also that, depending on the pH of ionophoresis, different proportions of inorganic phosphate and monophosphoglycerate were obtained from the diphosphoglycerate 'burst'. This curious effect is discussed below.

The inorganic phosphate which represented 90% of the decomposition products of the 2,3-diphosphoglycerate after ionophoresis at pH 3·5 (see Fig. 5*a*) did not seem to be present as such in the incubation mixture before ionophoresis was carried out. This was shown as follows. If after incubation of the enzyme with the labelled cofactor (2 min. in the cold) non-radioactive 3-phosphoglycerate was added and the products were separated by ionophoresis, no inorganic phosphate was obtained. This was true when the fractionation was carried out at pH 3·5 or at pH 6·5 (Fig. 6). However, when added inorganic [^{32}P]phosphate was incubated with 3-phosphoglycerate and enzyme, all the radioactivity was recovered as inorganic phosphate, and no incorporation into esters was detected. Since the

radioactivity associated with the active enzyme exchanges with the substrate, whereas inorganic [^{32}P]phosphate does not, the above results indicate that hydrolysis takes place during or after the denaturation process occurring at the drying stage before ionophoresis.

The method of analysis shown in Fig. 6 differentiates between two types of phosphate. The one present as protein-bound ^{32}P was incorporated into 3-phosphoglycerate before the ionophoresis. The ^{32}P present as inorganic phosphate was not. When the incubation of 2,3-diphosphoglycerate and enzyme was carried out for long periods and at room temperature (as in Fig. 4), inorganic phosphate was produced which could not be incorporated into the substrate by an incubation with 3-phosphoglycerate before fractionation as described in Fig. 6. The latter was a much slower process than the 'burst' hydrolysis, and corresponded to the linear phase of the phosphatase activity of Fig. 4.

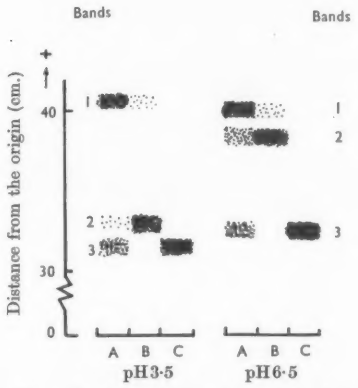


Fig. 6. Diagram of the radioactive products obtained by incubating phosphoglyceromutase and radioactive 2,3-diphosphoglycerate. A, Enzyme and ^{32}P-labelled cofactor (fivefold excess of cofactor) incubated for 2 min. in the cold (0·05 M-tris buffer, pH 7·0). B, Same as A further incubated with excess of non-radioactive 3-phosphoglycerate. C, Enzyme incubated with inorganic [^{32}P]phosphate and 3-phosphoglycerate. In all cases samples of the incubation mixture were applied to a sheet of Whatman 3MM paper and dried down. Electrophoresis (30 v/cm.) was carried out for 2 hr. at pH 6·5 and for 3 hr. at pH 3·5. Band 1, 2,3-diphosphoglycerate; band 2, monophosphoglycerate; band 3, inorganic phosphate.

Unequal amounts of inorganic phosphate and monophosphoglycerate were observed during the slow hydrolysis of the diphosphate in the presence of the enzyme. This difference could be increased substantially by increasing the enzyme/cofactor ratio and allowing the reaction to go to completion. This is shown in Table 1. It can be seen that, as the enzyme/cofactor ratio increased, more radioactivity was recovered as inorganic phosphate and less as monophosphoglycerate. That this difference is not due to subsequent hydrolysis of the monophosphoglycerate was shown in a control experiment in which the hydrolysis of the 3-phosphoglycerate was measured. The amount of inorganic phosphate formed at the expense of the monophosphoglycerate after incubation with the enzyme was not higher than the amount present in the controls (Table 2). These results suggest that the phosphatase activity involves a stoicheiometric hydrolysis of the diphosphoglycerate into inorganic phosphate plus monophosphoglycerate only when the enzyme concentration is negligible as compared with the diphosphoglycerate concentration.

DISCUSSION

To explain the presence of [^{32}P]phosphoserine in acid hydrolysates of rabbit-muscle crystalline phosphoglyceromutase (Pizer, 1960, 1962) as being

Table 1. *Effect of enzyme concentration on the slow hydrolysis of* 2,3-*di*[^{32}P]*phosphoglycerate*

2,3-Di[^{32}P]phosphoglycerate (5·4 mμmoles; $3{\cdot}9 \times 10^6$ counts/min./μmole) was incubated at room temperature with enzyme in 0·27 ml. of 0·05 M-tris buffer, pH 7·0. Samples were taken at the indicated times and analysed as indicated in Fig. 5(*b*). The inorganic [^{32}P]phosphate was not incorporated into the substrate when the samples were incubated with non-radioactive 3-phosphoglycerate.

Enzyme (mμmoles)	Ratio [enzyme]/[coenzyme]	Incubation time (hr.)	Radioactivity: Inorganic phosphate (%)	Radioactivity: Monophosphoglycerate (%)	Difference
0·25	0·046	4	58·5	41·5	17·0
0·25	0·046	20	58·8	41·2	17·6
0·5	0·093	20	67·9	32·1	35·8
1·0	0·185	20	74·6	25·4	49·2
3·5	0·66	20	77·7	22·3	55·4

Table 2. *Failure of phosphoglyceromutase to hydrolyse 3-phosphoglycerate*

[^{32}P]Diphosphoglycerate (6 mμmoles; $1{\cdot}2 \times 10^4$ counts/min./mμmole), 76 mμmoles of 3-phosphoglycerate and 10 mμmoles of enzyme were incubated at room temperature for 2 min. in 0·05 M-tris buffer, pH 7·0. Three samples were taken and treated as indicated. The radioactivity associated with the 3-phosphoglycerate fraction and the inorganic phosphate fraction were determined. The calculated ratio [^{32}P]monophosphoglycerate/inorganic [^{32}P]phosphate, as expected from 'burst' hydrolysis, is 6.

Sample	Treatment	Radioactivity (counts/min.) 3-Phospho-glycerate	Radioactivity (counts/min.) Inorganic phosphate	Ratio monophospho-glycerate / inorganic phosphate
A	Incubated for 20 hr. at room temperature	657	103	6·4
B	10 min. in boiling-water bath and 20 hr. at room temperature	935	187	5·0
C	20 hr. at −20°	675	99	6·8

due to a contamination with phosphoglucomutase, one must assume that: (1) crystalline phosphoglyceromutase is contaminated with phosphoglucomutase, and (2) 2,3-di[^{32}P]phosphoglycerate is contaminated with glucose 1,6-di[^{32}P]phosphate.

In the crystalline enzyme that we used we showed that the first condition was fulfilled and this was probably true also in the above-mentioned experiments (Pizer, 1960), since no special precautions were taken to avoid phosphoglucomutase contamination. The second condition requires that glucose 1,6-diphosphate is not separated from 2,3-diphosphoglycerate by chromatography in the propanol–ammonia–water system used for the separation of the phosphoesters. This was tested by us and shown to be the case. The purification of the diphosphate ester by paper electrophoresis reported in this paper yields a product which is free of such impurity. With this purified coenzyme no acid-stable phosphoenzyme could be obtained.

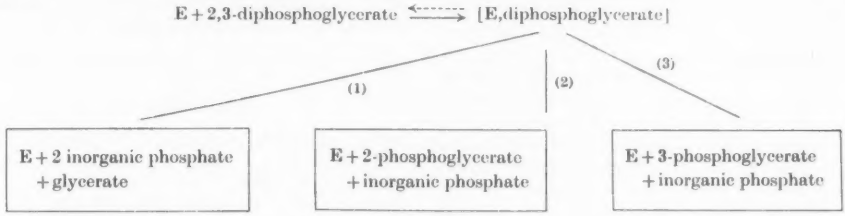
The presence of acid-labile ^{32}P in rabbit-muscle phosphoglyceromutase after incubation with its coenzyme was reported by Grisolia *et al.* (1961). Our results confirm this report. Furthermore, the methods applied in this work permitted a closer study of the nature of this weakly bound ^{32}P responsible for the diphosphoglycerate 'burst' and its relation to the phosphatase activity.

The diphosphoglycerate decomposition 'burst' cannot be attributed to the previously described diphosphoglycerate-phosphatase activity (Rodwell *et al.* 1957; Joyce & Grisolia, 1958) since: (a) In the linear phase hydrolysis of 1 mole of cofactor/mole of enzyme required (see Fig. 4) about 2 hr. at room temperature, whereas the 'burst' hydrolysis of 1 mole of cofactor/mole of enzyme was complete after only 2 min. incubation in an ice bath. (b) Under identical incubation conditions the decomposition products consistently depended on the treatment after application on to the paper. Unpublished results indicated that, after ionophoresis at pH 9, a high proportion of monophosphoglycerate was obtained (as at pH 6·5, see Fig. 5), whereas ionophoresis at pH 2·0 gave negligible amounts of it (as at pH 3·5, see Fig. 5). (c) The stoicheiometry of the 'burst' hydrolysis strongly suggested a specific effect. Any excess of coenzyme, under the conditions at which 'burst' hydrolysis was studied, was recovered as such. (d) After 2 min. in the cold there was no detectable inorganic [^{32}P]phosphate in the incubation mixture, as judged by its ability of being incorporated into the substrate.

We explain the diphosphoglycerate 'burst' by the properties of the intermediary enzyme–coenzyme complex, in which the phosphoester bonds of the diphosphoglycerate have lost their stability. It must be emphasized that the ionophoretic technique which we have used involved denaturation (the material is dried on the paper before the run), which could play an important role in the hydrolysis. Both bonds in diphosphoglycerate are known to resist very strong acid conditions without being hydrolysed (Greenwald, 1925), and the extreme lability of those bonds in the enzyme–coenzyme complex indicates a distortion effect due to binding to the protein. This lability in the denatured complex (as well as in the native complex, see below) explains why previous workers did not find bound co-enzyme in the mutase preparations (Rodwell *et al.* 1957). In fact the coenzyme bound to the enzyme is not a diphosphoglycerate ester in the chemical sense, but an enzyme–coenzyme intermediate in which the chemical properties of the ester bonds are no longer those of the molecular components.

The theoretically possible pathways of the decomposition of the enzyme–coenzyme complex which do not lead to formation of a phosphoenzyme intermediate, are shown in Scheme 1.

The possibility, however, of other pathways is not excluded. When ionophoresis is carried out on the



Scheme 1.

denatured complex at pH 6·5, pathways (2) and/or (3) could account for the products. Since under our conditions of analysis no distinction between 2-phosphoglycerate and 3-phosphoglycerate is possible we cannot say whether pathway (2), (3) or both are followed at pH 6·5. However, the results obtained in Fig. 5(*b*) suggest that, at this pH, pathway (2) might be preferred for the following reason. If the 2,3-diphosphoglycerate were homogeneously labelled, the radioactivity of the products inorganic phosphate and 2-(or 3-)phosphoglycerate ought to be the same (if they were produced in one to one molar ratio), or higher for inorganic phosphate if glyceric acid was also formed (pathway 1). To find a higher value for the monophosphoglycerate as shown in Fig. 5 was very puzzling at first. However, as reported by Prankerd & Altman (1954), the incorporation of ^{32}P into the 2,3-diphosphoglycerate by the erythrocytes, under the conditions used in this work to prepare the coenzyme, gave a diphosphoglycerate in which the specific radioactivity of the phosphate in position 2 is higher than that of position 3. The slightly but consistently higher radioactivity in the monophosphoglycerate fraction than in the inorganic phosphate fraction after ionophoresis at pH 6·5 (Fig. 5), suggests therefore a preference for pathway (2). A closer study of these effects requires the separation of the two monophosphate esters.

On the other hand, under the pH 3·5 conditions (Fig. 5*a*), most of the radioactivity was recovered as inorganic phosphate, suggesting that pathway (1) was preferred.

The results presented in this paper indicate that the specific hydrolysis of the coenzyme occurs in two clearly different steps. The 'burst' hydrolysis due to the destruction of the enzyme–coenzyme complex is negligible only when the ratio enzyme/coenzyme is negligible and does not represent a true phosphatase activity, since it is observed only after enzyme denaturation. However, after longer incubations a true diphosphoglycerate-phosphatase activity can be detected. This activity produces inorganic phosphate which is no longer in an exchangeable state. This phosphatase activity does not seem to be in itself a single reaction, since the stoicheiometry of the reaction does not follow a simple pattern and is influenced by the enzyme concentration (Table 1). This lack of stoicheiometry should not be confused with the lack of stoicheiometry during the burst reaction since (a) after 20 hr. there is no ^{32}P present which can be incorporated into the substrate and (b) there is an excess of inorganic phosphate produced over the amount of monophosphoglycerate, which is the opposite effect to the one observed during the 'burst' hydrolysis on ionophoresis at pH 6·5. In fact Table 1 suggests that when diphosphoglycerate and native enzyme are incubated for long periods, two simultaneous but independent phosphatase activities are superimposed. One corresponds to pathway (1) and could be due to the instability of the enzyme–coenzyme complex. Thus:

$$\text{E} + \text{2,3-diphosphoglycerate} \rightarrow [\text{E} - \text{diphosphoglycerate}] \rightarrow \text{E} + \text{2 inorganic phosphate} + \text{glycerate} \qquad (1)$$

The second phosphatase activity is the hydrolysis of the diphosphoglycerate giving inorganic phosphate and monophosphoglycerate as reported by Rodwell *et al.* (1957) for the yeast enzyme according to equation (2).

$$\text{2,3-Diphosphoglycerate} + \text{enzyme} \rightarrow \text{inorganic phosphate} + \text{2-(or 3-)phosphoglycerate} \qquad (2)$$

When there is less diphosphoglycerate than enzyme, reaction (2) is mostly suppressed, owing to the very low concentration of free 2,3-diphosphoglycerate, and the main products of the hydrolysis are those of equation (1) (Table 1). When there is a great excess of diphosphoglycerate the reaction proceeds mostly as shown by equation (2). Since the values of Fig. 4 were determined while there was still a great excess of diphosphoglycerate present, the linear phase of that Figure measures within experimental error the velocity of reaction

(2). This second phosphatase activity might be due to contamination with a specific 2,3-diphosphoglycerate phosphatase.

The method used to disclose the presence of the intermediary complex may prove of more general use in other cases in which such complexes have been postulated. The possibility that the hydrolysis of the enzyme–coenzyme complex could be responsible for some of the observed behaviour in some other mutases should be considered. Kinetic data of phosphoglucomutase (Bodansky, 1961) have suggested to Cleland (1963*a*,*b*) that the phosphoenzyme is not an intermediate in the reaction. Furthermore, recent results (Ray & Roscelli, 1964) have cast some doubt on the role of a phosphoenzyme intermediate in this reaction. If, as proposed in this paper for phosphoglycerate mutase, the transfer occurs directly via an enzyme–coenzyme complex in which the phosphate ester bonds become distorted, further handling could lead to the formation of inorganic phosphate or to a stable covalent bond with the substrate moiety and/or with an active residue of the protein. A distorted enzyme–glucose diphosphate complex could give a phosphoenzyme with its phosphate attached to the serine in the sequence Thr-Ala-Ser-His-[Asp or Asp(NH_2)].

Most of the experimental work described in this paper was done in the División de Biología Molecular, Instituto Nacional de Microbiologia, Buenos Aires, Argentina. We are grateful to Dr I. Pirosky for his unfailing support and to the Consejo Nacional de Investigaciones Cientificas y Técnicas (R. Argentina) for a grant.

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Some Observations on the Biosynthesis of the Plant Sulpholipid by *Euglena gracilis*

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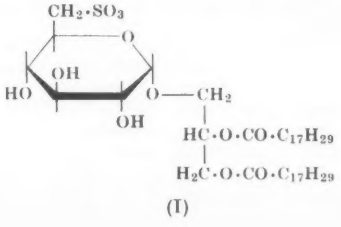
(*Received* 1 *July* 1965)

1. DL-Cysteine decreases the uptake of $^{35}SO_4^{2-}$ by *Euglena gracilis* but does not decrease the relative incorporation of the isotope into sulpholipid; cysteic acid, on the other hand, does not affect the uptake of $^{35}SO_4^{2-}$ but does dilute out its incorporation into the sulpholipid. 2. Both L-[^{35}S]cysteic acid and DL-+*meso*-[3-^{14}C]cysteic acid appear almost exclusively in 6-sulphoquinovose. 3. Molybdate inhibits the incorporation of $^{35}SO_4^{2-}$ into sulpholipid but not its uptake into the cells; this suggests that adenosine 3′-phosphate 5′-sulphatophosphate may be concerned with the biosynthesis of sulpholipid, and it was shown to be formed by chloroplast fragments. 4. An outline scheme for sulpholipid biosynthesis based on these observations is discussed.

Benson, Daniel & Wiser (1959) first showed that ^{35}S in the form of $^{35}SO_4^{2-}$ was rapidly incorporated into the lipids of green algae, photosynthetic bacteria and the leaf tissue of many higher plants. The structure of the sulpholipid involved was eventually shown to be 6-deoxy-6-sulpho-α-D-glucopyranosyl diglyceride [6-sulpho-α-D-quinovosyl diglyceride (I) (Daniel *et al.* 1961)]. The major fatty acid components are palmitic acid and linolenic acid (O'Brien & Shibuya, 1964). The major portion of the sulpholipid is present in the chloroplasts of algae and higher plants (Davies, Mercer & Goodwin, 1965), and the same lipid was present in all tissues (maize, runner beans, *Euglena gracilis* and cambial tissue culture of Paul's scarlet rose) examined by Davies *et al.* (1965).

The existence of 6-sulphoquinovose in the molecule raises a number of important questions of biosynthesis. We have investigated some aspects of origin of the sulphoquinovose molecule from DL-[^{35}S]cysteine, DL-[2-^{14}C]cysteic acid and L-[^{35}S]-cysteic acid as possible precursors of the sulphonic acid residue. In addition, the possibility of the existence of the sulphate-activating system which synthesizes adenosine 3′-phosphate 5′-sulphatophosphate from reduced sulphur has been examined.



(I)

EXPERIMENTAL

Organism. Euglena gracilis Z (1221–5Z) was obtained from The Collection of Algae and Protozoa, Botany School, Cambridge. It was maintained on slopes prepared from agar (1·5%, w/v) and yeast extract (Difco; 0·2%, w/v) in distilled water. It was cultured autotrophically on the medium of Brawerman & Chargaff (1959) and on the heterotrophic medium of Wolken (1961). Conical flasks (250ml.) containing 100ml. of medium were incubated at 18° without shaking. The cells were harvested after 6 days' growth by centrifugation.

Extration of sulpholipid. The method of Davies *et al.* (1965) was used.

Chromatography of sulpholipid. The thin-layer system of Davies *et al.* (1965) was generally used but occasionally the two-dimensional paper system of Benson *et al.* (1959) was used for reference purposes.

Detection and assay of sulpholipid. The administration of ^{35}S to a tissue is by far the most sensitive and unambiguous means of detecting the plant sulpholipid. The radioactive spots on the thin-layer chromatograms were located by radioautography with X-ray film (Kodirex). In this respect thin layers are some 20 times as sensitive as paper chromatograms (Goodwin, 1964). The radioactivity in the sulpholipid spot was quantitatively assayed by eluting it with toluene, adding the extract to a scintillator dissolved in toluene (5g. of 2,5-diphenyloxazole and 0·3g. of 2,2-phenyl-5-phenyloxazole in 1l.) and counting it in a Packard Tri-Carb Scintillation Spectrometer series 314E. All samples were bleached with u.v. light to prevent quenching by any pigment present.

Isolation of 6-sulphoquinovose from the sulpholipid. To a sample of [^{35}S]sulpholipid in 1ml. of aq. 50% ethanol, 0·5ml. of 6N-HCl was added and the mixture heated at 100° for 30min. On cooling 2ml. of water was added and the solution extracted with 5ml. of diethyl ether followed by 5ml. of chloroform. The aqueous layer was evaporated to dryness *in vacuo* and the residue dissolved in 1ml. of water. It was chromatographed on paper with either phenol saturated with water at 23° (Benson *et al.* 1959) or a butanol–propionic acid–water system (Benson *et al.* 1959) The R_F of 6-sulphoquinovose was 0·05 in both solvents. The diethyl ether and chloroform fractions were bulked and the total activity present was measured in the Packard machine. This activity was taken as that present in the diglyceride.

Preparation of chloroplasts from Euglena. Crude chloroplasts were obtained by ultrasonic treatment at 2° (MSE–Mullard Ultrasonic Disintegrator) of intact cells suspended in the medium of Stumpf & James (1963). The time required for ultrasonic treatment depended on the type of cells used: etiolated cells were given two periods of 30sec. duration separated by an interval of 60sec.; cells grown heterotrophically in the dark had three bursts of 30sec., each separated by an interval of 60sec.; autotrophic cells were given one burst of 60sec. followed by two bursts of 30sec., each separated by an interval of 60sec. The intervals were necessary to prevent excessive rises in temperature around the probe. These were then purified in two ways. In the first the disrupted cells were centrifuged at 140*g* for 5min.; the supernatant was then centrifuged for a further 5min. to ensure complete sedimentation of intact cells. The resulting supernatant was then centrifuged at 1000*g* for 12min. to sediment the chloroplasts. The pellet was resuspended in 20ml. of Stumpf & James (1963) buffer and recentrifuged at 1000*g* for 10min. In the second method the density-gradient technique of James & Das (1957) was used. Chloroplast fragments were prepared either by suspending the pellet in 0·5mM-MgO_2 for 5min., recentrifuging at 1000*g* and resuspending in distilled water, or by ultrasonic treatment at 2° (two 15sec. bursts) followed by the usual centrifugation procedure.

Radioactive materials. L-[^{35}S]Cystine, DL+*meso*-[3-^{14}C]-cystine hydrochloride and $^{35}SO_4^{2-}$ (carrier-free) were obtained from The Radiochemical Centre, Amersham, Bucks. L-[^{35}S]Cysteic acid and DL+*meso*-[3-^{14}C]cysteic acid were synthesized by the oxidation of the corresponding cystines with Br_2 in the presence of 0·25% HCl (Friedmann, 1903). Purification was achieved by thin-layer chromatography on Kieselgel G plates with butanol–propionic acid–water (6:3:4, by vol.) as solvent.

RESULTS

Effect of L-*cysteine on incorporation of* $^{35}SO_4^{2-}$ *into sulpholipid.* It is well known that cysteine is metabolized by animal tissues via cysteic acid and that the sulphur is eventually released as SO_4^{2-}. If cysteine sulphur could be oxidized by *Euglena* in this way then the incorporation of $^{35}SO_4^{2-}$ into sulpholipid should be reduced in the presence of L-cysteine.

A 6-day culture (1l.) of *E. gracilis* grown autotrophically was harvested by centrifugation, and the cells were resuspended in 150ml. of fresh medium. To three Erlenmeyer flasks (250ml.) each containing 50ml. of medium were added 50ml. of the suspension; $^{35}SO_4^{2-}$ was added to each flask (0·2mc/flask) and to two of the flasks were added 0·05g. and 0·5g. of DL-cysteine respectively. The flasks were incubated for 18hr. The cells were harvested and washed with 4 vol. of water to remove any free $^{35}SO_4^{2-}$. Each batch of cells was suspended in a known volume of water and samples were counted on a planchet with a Nuclear–Chicago D47 gas-flow detector. From this the uptake of $^{35}SO_4^{2-}$ in each flask could be calculated. The amount of [^{35}S]sulpholipid formed by the cells in each flask was determined as described in the Experimental section. The results (Table 1) indicate that although the higher concentration of DL-cysteine inhibits the uptake of $^{35}SO_4^{2-}$ by the cells the relative incorporation into the sulpholipid is very considerably increased; it changes from 1·53% in the absence of DL-cysteine to 27·62% in the presence of 0·5g. of DL-cysteine. It is evident from this experiment that, whatever the explanation of the effect of cysteine on $^{35}SO_4^{2-}$ uptake, there is no indication that the sulphur of cysteine is converted into SO_4^{2-} in sufficient amounts to dilute out the incorporation of $^{35}SO_4^{2-}$ into sulpholipid.

Effect of L-*cysteic acid on incorporation of* $^{35}SO_4^{2-}$ *into sulpholipid.* In animal tissue L-cysteic acid undergoes transamination with α-oxoglutarate and oxaloacetate to form β-sulphonylpyruvate (Cohen, 1940). The latter could be an intermediate in sulphoquinovose biosynthesis, and if so L-cysteic acid might by virtue of its conversion into β-sulphonylpyruvate dilute out the incorporation of $^{35}SO_4^{2-}$ into sulpholipid. On the other hand, the same result would be expected if SO_4^{2-} was liberated from L-cysteic acid. An experiment similar to that described for DL-cysteine in the previous section was set up and it will be seen (Table 2) that, in contrast with DL-cysteine, L-cysteic acid had no

Table 1. *Effect of* DL-*cysteine on the uptake and incorporation of* $^{35}SO_4^{2-}$ *into the sulpholipid in* Euglena gracilis

Autotrophic cultures (6 days) were incubated for 18hr. with $^{35}SO_4^{2-}$ (0·2mc/flask) in the presence or absence of DL-cysteine.

Amount of DL-cysteine added (g./flask)	$^{35}SO_4^{2-}$ taken up by cells (disintegrations/min.)	$^{35}SO_4^{2-}$ in sulpholipid (disintegrations/min.)	Incorporation into sulpholipid of $^{35}SO_4^{2-}$ taken up (%)
0	$7·2\times10^5$	$1·1\times10^4$	1·53
0·05	$6·7\times10^5$	$1·9\times10^4$	2·84
0·5	$2·1\times10^4$	$5·8\times10^3$	27·62

Table 2. *Effect of* L-*cysteic acid on the uptake and incorporation of* $^{35}SO_4^{2-}$ *into the sulpholipid in* Euglena gracilis

Autotrophic cultures (6 days) were incubated for 18 hr. with $^{35}SO_4^{2-}$ (0·5 mc/flask) in the presence or absence of L-cysteic acid.

Amount of L-cysteic acid/flask (g.)	$10^{-9} \times$ $^{35}SO_4^{2-}$ taken up by cells (disintegrations/min.)	$10^{-6} \times$ $^{35}SO_4^{2-}$ in sulpholipid (disintegrations/min.)	Incorporation into sulpholipid of $^{35}SO_4^{2-}$ taken up (%)
0	4·15	4·16	0·100
0·01	4·20	4·15	0·098
0·03	4·45	3·69	0·083
0·05	4·38	3·09	0·075
0·10	3·65	1·85	0·050

Table 3. *Comparison of the uptake of* $^{35}SO_4^{2-}$, L-[^{35}S]*cysteic acid* and DL + meso-[3-^{14}C]*cysteic acid into the sulpholipid in* Euglena gracilis

Experimental conditions were as outlined in Table 2.

Label added	Total activity in cells (disintegrations/min.)	Activity present in sulpholipid (disintegrations/min.)	Incorporation into sulpholipid of label taken up (%)
$^{35}SO_4^{2-}$	$1·15 \times 10^9$	$3·08 \times 10^6$	0·26
L-[^{35}S]Cysteic acid*			
(*a*)	$1·27 \times 10^7$	$2·91 \times 10^5$	2·29
(*b*)	$1·79 \times 10^7$	$5·16 \times 10^5$	2·88
DL + *meso*-[3-^{14}C]-Cysteic acid	$9·93 \times 10^5$	$2·64 \times 10^4$	2·66

* Separate experiments.

effect on the uptake of $^{35}SO_4^{2-}$ by *Euglena* except to a slight extent at the very highest concentration; it did, however, markedly reduce the incorporation of $^{35}SO_4^{2-}$ into the sulpholipid and this reduction increased with increased concentration of L-cysteic acid.

Incorporation of L-[^{35}S]*cysteic acid and* DL + meso-[3-^{14}C]*cysteic acid into sulpholipid.* To distinguish between the two possible explanations suggested above, of the ability of L-cysteic acid to dilute out the incorporation of $^{35}SO_4^{2-}$ into sulpholipid, an experiment was carried out to compare the incorporation of L-[^{35}S]cysteic acid and DL + *meso*-[3-^{14}C]cysteic acid with that of $^{35}SO_4^{2-}$. The results, collected in Table 3, demonstrate clearly that, when the cysteic acid is labelled with ^{35}S or ^{14}C in C-3, it is ten times more effectively incorporated into sulpholipid than is $^{35}SO_4^{2-}$ under the same conditions. Two samples of the sulpholipid produced in the presence of DL + *meso*-[3-^{14}C]cysteic acid were degraded and the activity was determined in the 6-sulphoquinovose and in the diglyceride residue. In both cases almost all the activity recovered was in the sulphoquinovose (Table 4).

Table 4. *Distribution of radioactivity in the sulpholipid of* Euglena gracilis *grown in the presence of* DL + meso-[3-^{14}C]*cysteic acid*

The sulpholipid from the experiment reported in Table 3 was degraded as described in the Experimental section.

	Total activity in sample (disintegrations/min.)	Activity in 6-sulphoquinovose (disintegrations/min.)	Activity in diglyceride residue	Activity recovered in 6-sulphoquinovose (%)
(*a*)	1240	922	171	84·5
(*b*)	1000	757	33	95·7

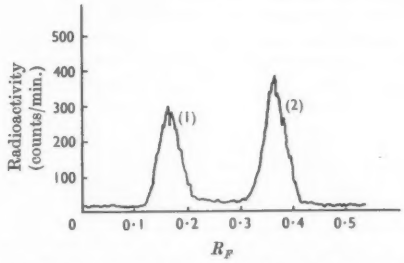


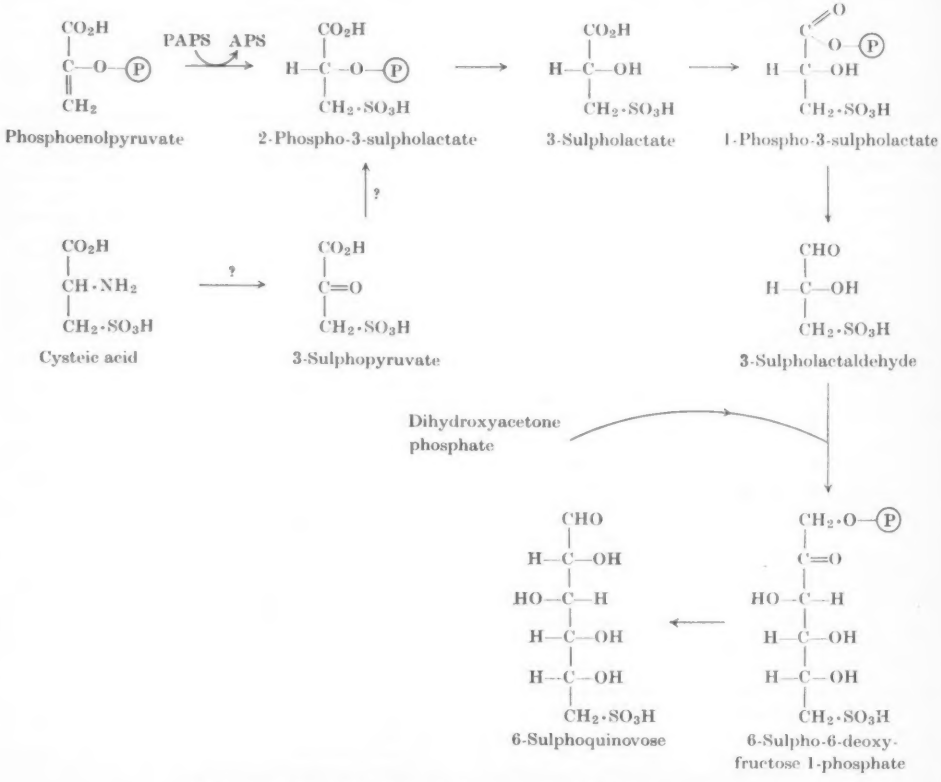
Fig. 1. Formation of adenosine 3′-phosphate 5′-sulphatophosphate (1) from ATP and $^{35}SO_4^{2-}$ (2) by *Euglena* chloroplast fragments. Reaction mixture (Asahi, 1964) was incubated with fragments of chloroplasts prepared by density-gradient centrifugation. Nucleotide extract was run on paper with propanol–ammonia–water (6:3:1, by vol.) as solvent and scanned for radioactivity.

Table 5. *Effect of* MoO_4^{2-} *on the uptake of* $^{35}SO_4^{2-}$ *by* Euglena gracilis *and its incorporation into sulpholipid*

Experimental conditions were as described in Table 2.

Concn. of MoO_4^{2-} (mM)	$10^{-6} \times$ Total uptake of $^{35}SO_4^{2-}$ (disintegrations/min.)	$10^{-4} \times$ Total activity in sulpholipid (disintegrations/min.)	Incorporation into sulpholipid of $^{35}SO_4^{2-}$ taken up (%)
0	3·78	5·78	1·53
0·03	3·52	4·40	1·25
0·3	3·97	3·61	0·91
3	4·67	3·58	0·77

Synthesis of adenosine 3′-phosphate 5′-sulphatophosphate by chloroplast fragments of Euglena gracilis. If 'active sulphur' (adenosine 3′-phosphate 5′-sulphatophosphate) were concerned in sulpholipid biosynthesis then one would expect it to be formed in the chloroplast, which is the major site of sulpholipids. Chloroplasts were prepared and fragmented as described in the Experimental section. The fragments were suspended in 0·5ml. of water and 0·5ml. of reaction mixture (tris, pH 7·5, 25μmoles; magnesium chloride, 5μmoles; $^{35}SO_4^{2-}$, 0·2mc) was added. After incubation for 90min. in the absence of light at 30° the reaction was stopped by boiling. The nucleotides were extracted and purified by column chromatography (Asahi, 1964) and then chromatographed together with $^{35}SO_4^{2-}$ as marker on paper with the solvent, propanol–ammonia–water (6:3:1, by vol.), recommended by Asahi (1964). The resulting chromatograph was scanned for radioactivity with the Nuclear–Chicago Automatic chromatogram scanner. The formation of adenosine 3′-phosphate 5′-sulphatophosphate, as indicated by the R_F value (Asahi, 1964) of the major radioactive zone, was clearly demonstrated. A scan obtained with chloroplast fragments prepared by density gradient is indicated in Fig. 1. A very similar pattern was obtained with chloroplast fragments prepared by disruption of chloroplasts by placing them in magnesium chloride (0·5mM) for 5min., although a small peak corresponding to adenosine 5′-sulphatophosphate was usually noted in these preparations. Although Asahi (1964) could demonstrate formation of adenosine 5′-sulphatophosphate in spinach chloroplasts, he was never able to obtain adenosine 3′-phosphate 5′-sulphatophosphate. He attributed this to the lability in plants of the kinase necessary for the phosphorylation of adenosine 5′-sulphatophosphate.



Scheme 1. Possible pathway for biosynthesis of 6-sulphoquinovose.

Abraham & Bachhawat (1963) demonstrated the formation of adenosine 3′-phosphate 5′-sulphatophosphate in the supernatant obtained by centrifuging disrupted *E. gracilis* cells at 20000*g*; they did not investigate the chloroplast fragment.

Effect of MoO_4^{2-} *on incorporation of* $^{35}SO_4^{2-}$ *into sulpholipid by* Euglena gracilis. Wilson & Bandurski (1958) have shown that molybdate inhibits the formation of adenosine 5′-sulphatophosphate by the enzyme ATP sulphurylase (ATP–sulphate adenylyltransferase, EC 2.7.7.4). If molybdate inhibited the uptake of $^{35}SO_4^{2-}$ into sulpholipid, then it would suggest that APS, and thus adenosine 3′-phosphate 5′-sulphatophosphate, are on the route from SO_4^{2-} to sulpholipid.

Table 5 gives the result of an experiment which shows that, whereas MoO_4^{2-} at concentrations of up to 3mM has no effect on the uptake of $^{35}SO_4^{2-}$ by *E. gracilis*, it does inhibit incorporation into sulpholipid; the inhibition amounts to about 50% at the highest concentration used.

DISCUSSION

It would appear from Table 1 that the sulphur from cysteine is not incorporated into sulpholipid by *E. gracilis*; that is the organism cannot oxidize the SH of cysteine to a sulphonic acid grouping such as exists in the sulpholipid and in cysteic acid. An indirect effect of added cysteine cannot be ruled out but it seems unlikely because Nissen & Benson (1964) showed that [3-^{14}C]cysteine is not incorporated into the sulpholipid in *Chlorella*. However, our experiments with [^{35}S]cysteic acid and [3-^{14}C]-cysteic acid show that cysteic acid can provide a C–S unit for sulpholipid biosynthesis. Benson & Shibuya (1961) have demonstrated the presence of sulpholactaldehyde, sulpholactic acid and sulphopropanediol in *Chlorella pyrenoidosa*. These observations suggest that the sulphur is added at a C_3 stage. The existence of adenosine 3′-phosphate 5′-sulphatophosphate in *Euglena* and the inhibition of $^{35}SO_4^{2-}$ uptake into sulpholipid by MoO_4^{2-} suggests that the compound may take part in sulpholipid biosynthesis, perhaps by forming a C–S linkage with phosphoenolpyruvate; the resulting 2-phospho-3-sulpholactate could eventually be converted into 3-sulpholactaldehyde, which could undergo an aldol condensation with dihydroxyacetone phosphate to form 6-sulpho-6-deoxyfructose 1-phosphate, which on isomerization would yield 6-sulphoquinovose (Scheme 1). Cysteic acid could then enter this pathway via 3-sulphopyruvate, which, as stated above, is known to be formed by transamination in animals (Cohen, 1940). It should be emphasized that cysteic acid is not envisaged as a normal intermediate in sulpholipid biosynthesis.

We thank the Agricultural Research Council for financial support for this project.

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Metabolic Changes in the Skin of Rats of Various Ages

OXYGEN CONSUMPTION AND UPTAKE OF GLUCOSE

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(*Received* 13 *July* 1965)

1. Parallel determinations of the oxygen consumption by skin slices with glucose or galactose as substrate and of the concentration of DNA of the skin of various ages of rats were carried out to study the metabolic changes of the skin with age. 2. Both the oxygen consumption and the DNA content of the skin decreased rapidly till the attainment of maturity; thereafter the rate of decrease in oxygen consumption was lower whereas the concentration of DNA was constant. The decrease in the oxygen consumption during the early developmental period is thus due to the loss of cells, but the decrease in the older rats may be due to a decline in the activity of the cells of the skin as a result of aging. 3. The uptake of glucose by the skin decreased with increasing age.

It is well established that in certain systems of the body cellular functions decline with age (Shock, 1961). According to Barrows (1956) the overall activity of tissue slices in general, as seen from oxygen consumption and anaerobic glycolysis, declines with age. We (Kanungo & Patnaik, 1964; Patnaik & Kanungo, 1965) have shown that the uptake of ascorbic acid by the skin of the rat declines with age, which suggests a direct relationship between ascorbic acid and the synthesis of collagen by the fibroblasts of the skin. Glucose has been shown to be necessary for collagen synthesis (Flanagan & Nichols, 1964) and also as a precursor of both hexosamine and hexuronic acid (Davidson, Woodhall & Baxley, 1962). Sobel, Wright & Gabay (1958) showed that the concentration of hexosamine in the skin declines with age. In view of the above observations, oxygen consumption by skin slices with glucose and galactose as substrates and the uptake of glucose by skin slices of rats of various ages were studied to evaluate the metabolic changes in the aging rat.

MATERIALS AND METHODS

Animals. The albino rats used were of Wistar strain and were kept at 24±2°. They were fed on standard Anidiet of the Chelsea Laboratories, Poona, India. They were also given gram (*Cicer arietinum*) on alternate days and tap water *ad lib.* The rats used for the study were 4, 12, 34, 45 and 65 weeks old.

Preparation of skin slices. The animals were killed by dislocation of the neck. Several pieces of the skin from the dorsal surface were cut immediately, hairs were plucked by hand and the skin was kept in ice-cold Krebs–Ringer phosphate–bicarbonate saline (Krebs & Henseleit, 1932) that had been oxygenated previously. Slices of the skin were weighed and then cut into thin sections with a razor blade. About 0·25g. of skin was used for each experiment.

Oxygen consumption by skin slices. The oxygen consumption by the skin slices was measured manometrically in an Aminco refrigerated Warburg respirometer. The total incubation medium including the skin slices was 3·0ml., which included 2·25ml. of Krebs phosphate–bicarbonate saline, pH7·4, and 0·5ml. of substrate (5mM), which was placed initially in the side arm. The centre well contained 0·2ml. of 20% (w/v) KOH. The temperature of incubation was 37° and the gas phase was air. The skin slices were placed in the main chamber and the flasks were equilibrated by shaking at 90 oscillations/min. for 15min. The substrate was then transferred to the main chamber and readings were taken at 10min. intervals for 60min. The oxygen consumption was expressed as ml. of O_2/g. dry wt./hr. and ml. of O_2/mg. of DNA/hr.

Glucose uptake by skin slices. The uptake of glucose by the skin slices was determined parallel with the measurement of oxygen consumption. The initial and the final concentrations of glucose in the incubation medium were determined to find out the amount of glucose taken up by the skin slices. The initial concentration of glucose was the same as that for the measurement of the oxygen consumption. Parallel controls that did not contain glucose were set up each time. Samples (1·0ml.) of the incubation medium were withdrawn from the Warburg flasks after the 1hr. incubation period and were mixed with equal volumes of cold 6% (w/v) trichloroacetic acid containing 0·1% (w/v) of Ag_2SO_4. Duplicate samples of the supernatant after centrifugation were taken for the determination of the final concentration of glucose in the incubation medium according to the method of Mendel, Kemp & Myers (1954). The extinction of the colour developed was read at 520mμ in a Beckman model DB spectrophotometer. The concentrations of glucose in the unknown samples

were determined from a standard curve which was linear and was drawn each time. The uptake of glucose was expressed as mg. of glucose/g. wet wt./hr. and mg. of glucose/g. dry wt./hr.

DNA content of the skin. Pieces of the skin from the dorsal surfaces of 1-day-old and 4-, 12-, 45- and 65-week-old rats were cut and the hairs were removed. Portions (1·0g.) of skin were ground in 10·0ml. of water in a precooled mortar containing a small amount of chemically pure sand. Then 5·0ml. of 10% (w/v) trichloroacetic acid was added to 2·0ml. of the resulting homogenate. The mixture was centrifuged at $6000g_{av.}$ at 0° for 10min. in an International refrigerated centrifuge. The precipitate was resuspended in 5·0ml. of 10% trichloroacetic acid and centrifuged at $6000g_{av.}$ for 10min. This precipitate was extracted twice with 10ml. of 90% (v/v) ethanol and was then suspended in 1·3ml. of water. The rest of the procedure for the determination of the concentration of DNA was the same as that of Schneider (1957). The extinction of the colour developed was measured at 600mμ. The calculations were based on the datum that 1·0μg. of DNA gives an extinction of 0·019 at 600mμ. The concentration of DNA was expressed as mg. of DNA/100g. dry wt. of skin.

Standard deviations of the mean and the levels of significance were calculated according to the method of Garret (1956).

RESULTS

Figs. 1 and 2 show that the oxygen consumption of the skin slices with glucose or galactose as the substrate declined sharply till 12 weeks of age, both on the basis of dry weight and DNA content of the skin. Thereafter the oxygen consumption decreased slowly until 65 weeks of age. The differences between the oxygen consumption by the skin of various age groups were highly significant.

Fig. 3 shows that the uptake of glucose by the skin slices decreased with age. The differences between the uptake by the skin of various age

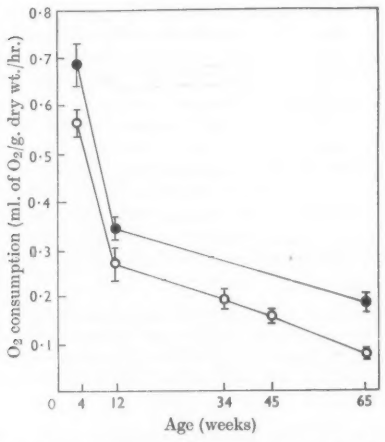

Fig. 1. Oxygen consumption of the skin of 4-, 12-, 34-, 45- and 65-week-old rats. Slices were incubated as described in the text. ●, With D-galactose as substrate (4-, 12- and 65-week-old female rats); ○, with D-glucose as substrate (4-, 12-, 34-, 45- and 65-week-old male rats). Each point represents the mean of seven experiments and the vertical bars represent ±S.D.

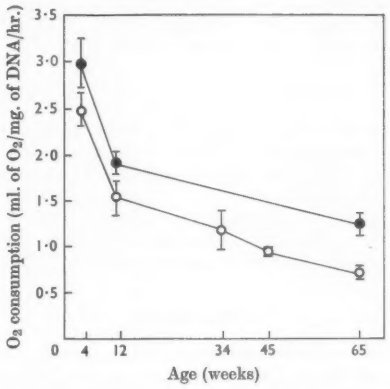

Fig. 2. Oxygen consumption by the skin of rats. Experimental details are given in the text. ●, With D-galactose as substrate (4-, 12- and 65-week-old female rats); ○, with D-glucose as substrate (4-, 12-, 34-, 45- and 65-week-old male rats). Each point represents the mean of seven experiments and the vertical bars represent ±S.D.

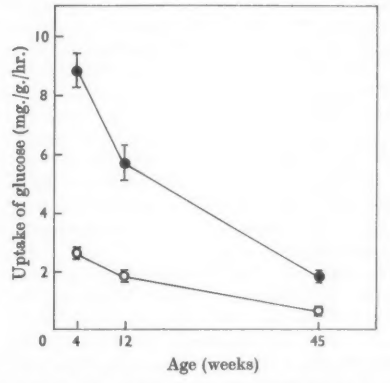

Fig. 3. Uptake of glucose by the skin slices of 4-, 12- and 45-week-old male rats. Experimental details are given in the text. ●, mg. of glucose/g. dry wt./hr.; ○, mg. of glucose/g. wet wt./hr. Each point represents the mean of seven experiments and the vertical bars represent ±S.D.

Table 1. *Concentration of DNA in the skin of rats of various ages*

Determinations were made as described in the text. Results are given as means ± S.E.M., with the numbers of animals in parentheses.

Age (days)	Concn. of DNA (mg./100g. dry wt.)	Significance of change (P)
1	69·19 ± 4·07 (3)	< 0·001
28 (4 weeks)	23·02 ± 1·23 (10)	< 0·01
84 (12 weeks)	18·27 ± 0·09 (4)	0·10
315 (45 weeks)	17·16 ± 0·54 (4)	0·05
455 (65 weeks)	15·49 ± 0·39 (6)	

groups were highly significant. Table 1 shows that the concentration of DNA in the skin declined rapidly during the growth period. The concentrations of DNA in the skin of 12- and 45-week-old rats were not significantly different. The DNA content of the skin of 65-week-old rats was slightly lower than those of 12- and 45-week-old rats.

DISCUSSION

The present studies show that oxygen consumption by the skin slices with glucose or galactose as the substrate is highest in the 4-week-old rats but declines rapidly as the animals reach maturity (12 weeks old). Thereafter the oxygen consumption decreases with increasing age (Figs. 1 and 2). Simultaneous determination of the DNA content of the skin also shows a rapid decline during the early development of the rat till it becomes 12 weeks old. After this the decline is insignificant (Table 1). Therefore the oxygen consumption of the skin in the early developmental period at least appears to be related to cell number. However, in the older rats this may not be so. Though there is no difference in the cell number between the skins of 12- and 45-week-old rats on the basis of DNA content, the oxygen consumption of skin of the older rats is significantly lower ($P < 0·001$) than that of the younger rats. The lower oxygen consumption by skin of the 45-week-old rats may thus be due to the decreased activity of the cells of the skin as a result of aging.

The oxidative system of the cells is impaired in ascorbic acid deficiency (King, 1950) and the concentration of ascorbic acid in the skin of rats declines with age (Kanungo & Patnaik, 1964). It is possible therefore that the lowering of concentration of ascorbic acid in the skin of rats may be responsible in some way for the decreasing oxygen consumption with age.

The uptake of glucose by the skin slices of rat declines with age (Fig. 3). Glucose is necessary for the synthesis of collagen in that the carbon skeletons of the amino acids are derived from it (Flanagan & Nichols, 1964). The higher uptake of glucose by the skin of 4-week-old rats as compared with that of older rats may be due to two reasons: (1) since the rate of collagen synthesis in the skin of rats remains high till the age of 5 weeks (Wirtschafter & Bentley, 1962), the demand for glucose may also be high during this period; (2) the fibroblasts may be metabolically more active during the rapid synthesis of collagen in the young rats and may require more energy, which is derived by the oxidation of glucose.

Glucose has also been shown to be incorporated into the mucopolysaccharides of cultivated connective tissue (Guha, Mukherjee, Balasubramanian & Bacchawat, 1964). Davidson *et al.* (1962) found that glucose serves as a precursor of glucosamine. Further, the concentration of hexosamine, which is a component of the mucopolysaccharide of the skin, declines with age (Sobel *et al.* 1958). Hence the decrease in the uptake of glucose by the skin may also be due to a lower rate of synthesis of hexosamine, which may affect the synthesis of mucopolysaccharide.

In most of the tissues studied by Shock (1960) and Barrows, Roeder & Falzone (1962) no significant change in the DNA content was found when young and adult rats were compared. Nikitin, Novikova & Zikalo (1960) determined the DNA content of the heart, kidney, intestine and liver of newborn and 1-, 3-, 12- and 24-month-old rats, and found that its concentration decreased till the age of 3 months, after which there was no change. Our results show that also in the skin the DNA content declines till the age of 12 weeks, after which there is no significant decrease (Table 1). This shows that cell loss occurs only during the early developmental period. After the attainment of maturity the rate of cell loss is insignificant. However, since the skin continues to expand even after maturity, this increase in the surface area may be due to increasing accumulation of collagen fibres and mucopolysaccharides in the intercellular spaces of the skin.

The authors are grateful to Professor S. P. Ray-Chaudhuri for laboratory facilities and encouragement. The Council of Scientific and Industrial Research, New Delhi, kindly awarded a research grant to M. S. K. and a Junior Research Fellowship to B. K. P.

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Oxidative Phosphorylation accompanying Oxidation of Short-Chain Fatty Acids by Rat-Liver Mitochondria

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(*Received* 31 *August* 1965)

1. The factors concerned in the estimation of P/O ratios when fatty acids are oxidized by rat-liver mitochondria have been assessed. 2. The oxidation of butyrate, hexanoate and octanoate is accompanied by ATP synthesis. At low concentrations of the fatty acids, P/O ratios approximately 2·5 are obtained. 3. Oxidative phosphorylation is uncoupled, respiratory control ratios are lowered and respiration is inhibited when the concentration of the fatty acid in the incubating medium is raised (to 5–10mM); octanoate is a more potent uncoupler than either hexanoate or butyrate. 4. Serum albumin and carnitine, either singly or in combination, protect the mitochondria from the effect exerted by the fatty acids. 5. The rate of oxidation of short-chain fatty acids in the presence of ADP is increased in the presence of carnitine.

The oxidation of fatty acids provides a major source of energy for mammalian tissues, but the experimental evidence which directly connects this process with ATP formation is limited. Kennedy & Lehninger (1949) have shown that the oxidation of octanoate in the presence of malate by rat-liver mitochondria is accompanied by extensive incorporation of ^{32}P from inorganic phosphate into ATP. Judah & Williams-Ashman (1951) have obtained P/O ratios 0·56 during the oxidation of octanoate by rat-liver mitochondria in the presence of succinate, and Lardy & Wellman (1952) have shown that the oxidation of octanoate to acetoacetate by such mitochondria gives P/O ratios 1·1–2·0. In the presence of added succinate, rat-kidney mitochondria gave P/O ratios 1·9–2·3 (Maley & Lardy, 1953) when octanoate was oxidized.

Fatty acids have been shown to uncouple oxidative phosphorylation occurring during the oxidation of other substrates (Scholefield, 1956; Hülsmann, Elliott & Slater, 1960) and such uncoupling can be prevented by the addition of serum albumin to the system (Lehninger & Remmert, 1959; Wojtczak & Wojtczak, 1960; Björntorp, Ells & Bradford, 1964). Carnitine has been shown to increase the rate of oxidation of long-chain fatty acids but has slight or no effect on those of short-chain length (Miller & Krake, 1962; Fritz, 1963). There is no information available on the relation of this compound to ATP production during the oxidation of fatty acids.

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In the present work factors affecting the estimation of P/O ratios obtained during the oxidation of fatty acids, both manometric and amperometric methods of measuring oxygen consumption being used, have been examined. P/O ratios for the oxidation of selected short-chain fatty acids by rat-liver mitochondria have been obtained in the presence and absence of serum albumin and carnitine.

EXPERIMENTAL

Materials

ADP was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and analysed for easily hydrolysable phosphate and total phosphate (Dounce, Rothstein, Beyer, Meier & Freer, 1948). The theoretical amount of phosphate was obtained. A further check by measuring its extinction at 259mμ confirmed the phosphate analysis (Chance & Williams, 1955). ATP was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; DL-carnitine hydrochloride from Mann Research Laboratories, New York, N.Y., U.S.A.; the fatty acids (British Drug Houses Ltd., Poole, Dorset) were distilled before their conversion into the potassium salts. Crystalline bovine serum albumin was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. It was dissolved in water (20%, w/v) and dialysed for 48hr. against 10 changes of distilled water (2l.) at 2° to remove possible traces of citrate. All other reagents used were of analytical grade.

Crystalline hexokinase (EC 2.7.1.1) (170000 units/g.: one unit is the activity of enzyme which utilizes 1μmole of ATP/min. at 38° in a medium of pH 7·4) was obtained from Sigma Chemical Co. The suspension in $(NH_4)_2SO_4$ soln. (3ml.) was dialysed against 1l. of

glucose solution (0·3M) at 2° for 24hr., or until no trace of NH_4^+ remained (up to 10 changes). The hexokinase was estimated by following, with a pH-stat (Radiometer, Copenhagen, Denmark), the rate of production of protons during the conversion of glucose into glucose 6-phosphate in the presence of ATP and Mg^{2+} at 38°. Yeast inorganic pyrophosphatase was prepared from dried baker's yeast (Heppel & Hilmoe, 1951).

Methods

The formation of ATP from ADP and inorganic phosphate associated with the oxidation of glutamate and fatty acids was followed by two methods.

Method 1: uptake of inorganic phosphate and manometric estimation of oxygen. Rat-liver mitochondria were prepared according to the method described by Hird & Symons (1962). The final mitochondrial pellet was suspended for use in the tris–KCl medium of Chappell & Perry (1954) at pH7·4 (4ml. of final mitochondrial suspension was obtained from 10g. of liver fresh wt.). The absence of sucrose in this medium allowed ketone bodies to be estimated without interference. Oxygen consumption was estimated in the Warburg apparatus at 38°. The flasks contained 0·3ml. of the mitochondrial suspension in a final volume of 3·0ml. In addition to substrates (10·0mM-butyrate, 7·5mM-hexanoate and 5·0mM- or 1·0mM-octanoate) the incubation mixture consisted of: potassium phosphate buffer, pH7·4 (23·3mM); tris–HCl buffer, pH7·4 (36·7mM); $MgCl_2$ (2·5mM); EDTA (mM); KCl (30mM); ATP (mM). Bovine serum albumin in the amounts indicated in the text and inorganic pyrophosphatase (in 0·5ml.) were added to the main compartment. The side arm contained glucose (final concn. 23·3mM) and hexokinase as indicated.

At the end of a 10min. equilibration period the contents of the side arm were added to the main compartment and the incubation was carried out with a gas phase of air. Oxygen consumption was followed until approximately 8μmoles were taken up, when the reaction was stopped by the addition of 0·3ml. of trichloroacetic acid (10%, w/v). The inorganic phosphate present before and after the addition of hexokinase was estimated by the method of Allen (1940). In each manometric experiment an estimate of glucose 6-phosphatase activity of the mitochondrial preparations was made to correct for the amount of phosphate cycled in this way. Glucose 6-phosphatase was estimated by following the production of inorganic phosphate from glucose 6-phosphate (10mM) in a medium similar to that used to determine the P/O ratios but low in phosphate (6·6mM). The K_m for liver glucose 6-phosphatase is approx. 2·5mM (Langdon & Weakley, 1957). Linearity of glucose 6-phosphate formation in the phosphorylating system can be assumed and as 30μmoles or more of glucose 6-phosphate were formed during the reaction period the enzyme was saturated for most of the time. The contribution up to saturation concentration was calculated from the K_m. The correction to the P/O ratio was never greater than 15%.

Inorganic pyrophosphatase activity in the system was followed by estimation of inorganic phosphate formed from pyrophosphate (20μmoles/3ml.), this being an excess over the amount of pyrophosphate produced in the system. The activity of the pyrophosphatase added to the incubation system was sufficient to hydrolyse 25μmoles of pyrophosphate/min. at 38°. Ketone-body production was estimated by the method of Hird & Symons (1959), with the subsequent modifications by Hird & Weidemann (1964), and fatty acid disappearance was estimated by steam-distillation and titration under CO_2-free conditions as described by Pennington (1952).

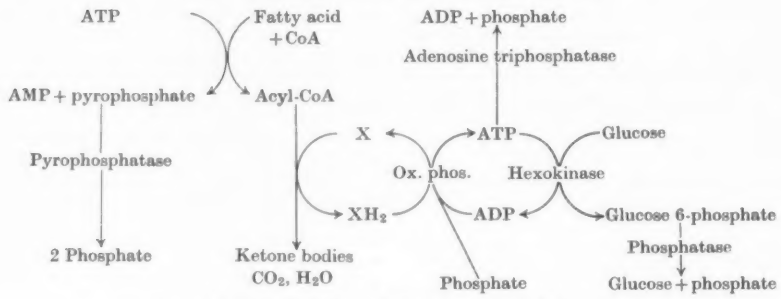
Method 2: amperometric estimation of oxygen consumption coupled to phosphorylation of ADP. Experiments were performed at 30° in a cell with capacity 1·5ml. An oscillating glass-covered electrode (Gilson Medical Electronics, Middleton, Wis., U.S.A.) with exposed platinum tip (0·6mm.), coupled to a Metrohm Polarecord type E 261 (Switzerland) amplifier and recorder, was used. The recorder was gain-controlled to give a pen deflexion of approximately 14cm. for the O_2 dissolved in the medium. The O_2 concentration in the medium was measured at −0·6v with a calomel reference electrode, and was determined by measuring the deflexion of the recorder pen with M-KCl saturated with air at 30° (*International Critical Tables*, 1928) followed by purging with O_2-free N_2, and directly comparing this deflexion with that obtained with the medium under the same conditions. Liberation of O_2 from H_2O_2 with catalase in the reaction mixture and in M-KCl (Dixon & Kleppe, 1965) indicated that with the oscillating electrode the activity of oxygen in both systems was the same within the limit of experimental error. The concentration of O_2 in the reaction mixture so determined was 0·46μg.atom/ml., which is comparable with 0·445μg.atom of oxygen in a similar system determined by Chappell (1964).

The reagents were added to the reaction cell by means of syringes. Rapid stirring of the system was accomplished by means of a small circular metal stirrer connected to a vibrator from an electric razor with a foot switch. Stirring for 1sec., after addition of reagents, was sufficient for complete mixing. The stirrer remained in the vessel during the experiment.

During the course of the incubation, contact between the platinum electrode and the incubating medium containing mitochondria led to slow poisoning of the electrode. This problem was overcome by renewing the electrode surface immediately before each run by burnishing it with fine carborundum paper.

Mitochondria were prepared from rat liver as before. In most of the experiments performed by this method, sucrose solution (0·3M) was used as the suspending medium instead of the tris–KCl medium of Chappell & Perry (1954). The mitochondria (from 5g. of liver fresh wt.) were suspended in 1·0ml. of 0·3M-sucrose solution, and 0·1ml. of the suspension was used each time; the composition of the reaction mixture was as described by Hird & Symons (1962). The final volume was 1·5ml. In some experiments, bovine serum albumin (1%, w/v) and DL-carnitine hydrochloride (in equivalent amounts to the substrate, below 1mM, and thereafter at 1mM) were also added to the incubating medium. To initiate ADP-dependent respiration, ADP (0·4μmole in 0·01ml.) was added to the reaction vessel with the aid of an Agla micro-syringe.

Mitochondria suspended in sucrose solution were used as soon as possible after preparation, and during the course of the experiment were stored in an ice bath at 0°. With each batch of mitochondria, and glutamate as substrate, it was possible to make approximately 20 estimations (approximately 2hr.) without apparent deterioration of respiratory control and P/O ratio. After this time some



Scheme 1. Ox. phos.: Oxidative phosphorylation.

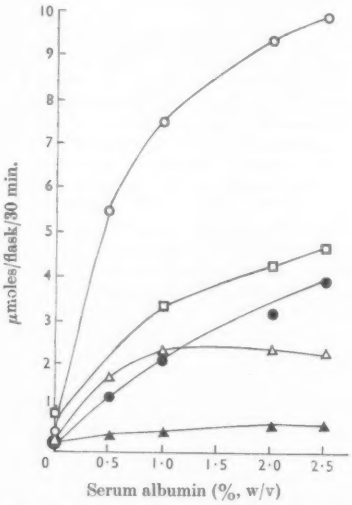


Fig. 1. Effect of serum albumin on the oxidation of butyrate by rat-liver mitochondria. Hexokinase (10 units) was added to each flask; other experimental details are given in the text. In the presence of butyrate (10mM): ○, oxygen consumption (measured manometrically); △, ketone-body production; □, butyrate disappearance. In the absence of added substrate: ●, oxygen consumption; ▲, ketone-body production.

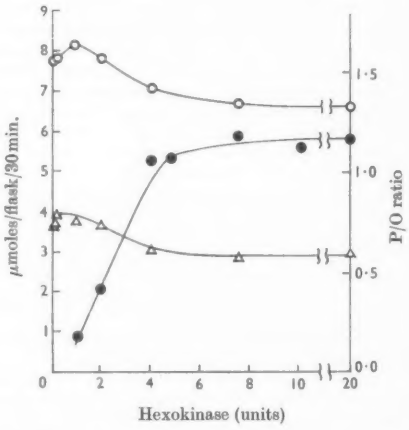


Fig. 2. Effect of hexokinase concentration on the oxidation of butyrate by rat-liver mitochondria. Butyrate (10mM) and serum albumin (1%, w/v) were added to each flask; other experimental details are given in the text. ○, Oxygen consumption (measured manometrically); △, ketone-body production; ●, P/O ratio (uncorrected).

loss of respiratory control became evident and increased progressively.

RESULTS

Method 1: uptake of phosphate and manometric estimation of oxygen

General principles of the method. The general reactions shown in Scheme 1 are involved or interfere in the determination of P/O ratios by this method.

Because the method is dependent on total inorganic phosphate disappearance in relation to oxygen consumption, the existence of processes returning phosphate to the medium will lower the P/O ratios observed. The magnitude of possible corrections to be applied must therefore be assessed. To do this it is necessary to estimate ATP consumed in the activation reaction (by measuring the disappearance of fatty acid) and to estimate pyrophosphatase, adenosine-triphosphatase and glucose 6-phosphatase activities of the system. The

ability of hexokinase to compete with the mitochondrial adenosine triphosphatase for the ATP formed, and the possible uncoupling effects of the fatty acids, have also to be assessed.

ATP utilization during the activation reaction and the status of endogenous pyrophosphatase. Determination of the fatty acid oxidized during the course of each experiment enables an estimate of the ATP consumed in the esterification reaction with coenzyme A to be made. Provided that the inorganic pyrophosphate so formed is either not hydrolysed or completely hydrolysed during the incubation, a simple correction can be applied. In the present system, pyrophosphatase activity for complete hydrolysis was sometimes found to be insufficient. To ensure that the pyrophosphate formed was completely hydrolysed, excess of inorganic pyrophosphatase prepared from yeast (Heppel & Hilmoe, 1951) was added. The amount of phosphate returned to the medium via ATP consumed by the thiokinase reaction was corrected for on the basis of 2 moles of phosphate/mole of fatty acid esterified.

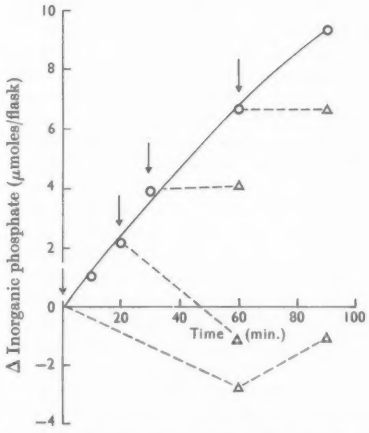


Fig. 3. Effect of hexokinase on the effective adenosinetriphosphatase activity of rat-liver mitochondria. The incubating medium contained ATP (10μmoles) and other constituents as given in the text (including 1% serum albumin), but there was no added substrate. Inorganic phosphate liberated from ATP: ○, in the absence of added hexokinase; △, in the presence of 10 units of hexokinase added at various time-intervals as indicated by the arrows.

Correction for glucose 6-phosphatase. The glucose 6-phosphatase of rat liver is associated with the microsome fraction (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) and, even when great precautions were taken for the complete removal of the fluffy layer from the mitochondrial preparations, it was not always possible to free the mitochondria of residual activity. It was therefore necessary to estimate glucose 6-phosphatase activity in the system for each batch of mitochondria. This corresponded to a correction of 0–15% in the final P/O ratio.

Effect of bovine serum albumin. Fig. 1 shows that the rate of oxidation of butyrate in the system was markedly stimulated by serum albumin. This stimulation fell off at about 1% (w/v), especially with ketone-body production. Fig. 1 also shows that serum albumin, or its bound fatty acids, acted as a substrate in the absence of butyrate but was not a good precursor of ketone bodies. As the rate of oxidation of butyrate was inconveniently low in the absence of serum albumin, this protein was always added to the system used. A concentration of 1% (w/v) was considered appropriate. It is likely that some suppression of endogenous oxidation occurred in the presence of fatty acid substrate (Hird & Symons, 1959), but this aspect of the system has not been investigated further.

Effect of hexokinase concentration. Fig. 2 shows

Table 1. P/O *ratios from oxidation of fatty acids*

Rat-liver mitochondria suspended in the tris–KCl medium of Chappell & Perry (1954) were incubated in Warburg flasks for 30–40 min. at 38° in 3·0 ml. of medium as described in the text. All flasks contained hexokinase (20 units) and serum albumin (1%, w/v). The results are expressed in μmoles/0·3 ml. of mitochondrial suspension. Standard deviations are given in parentheses. Corrected P/O ratio (1) indicates correction for ATP consumed in the activation reaction; corrected P/O ratio (2) includes additional correction for glucose 6-phosphatase activity.

Additions	No. of expts.	Oxygen uptake (μmoles)	Fatty acid metabolized (μmoles)	Ketone bodies formed (μmoles)	P/O ratio: Uncorrected	P/O ratio: Corrected (1)	P/O ratio: Corrected (2)
10mM-Butyrate	4	7·2 (±0·9)	3·4 (±0·4)	1·9 (±0·3)	1·5 (±0·1)	1·9 (±0·1)	2·1 (±0·1)
7·5mM-Hexanoate	4	7·1 (±0·8)	2·4 (±0·3)	1·8 (±0·1)	1·5 (±0·1)	1·8 (±0·2)	2·0 (±0·1)
5mM-Octanoate	4	6·0 (±2·6)	1·4 (±0·8)	1·4 (±0·8)	0·5 (±0·4)	0·7 (±0·5)	0·8 (±0·5)

that the addition of 6 units of hexokinase was sufficient for the determination of P/O ratios. At this concentration, however, there was a slight fall in the rate of fatty acid oxidation and ketone-body formation. The high rate of oxygen consumption in the absence of hexokinase, and the quite small stimulation in its presence, indicates uncoupling of oxidative phosphorylation by the fatty acid and/or stimulation of some type of mitochondrial adenosine-triphosphatase activity. The same stimulation occurs in the absence of a phosphate acceptor, suggesting that the high rate is not due to the presence of endogenous hexokinase and adenosine triphosphatase.

To investigate possible competition for ATP between adenosine triphosphatase and the hexokinase system, the latter was added at various time-intervals to a mitochondrial system actively hydrolysing ATP. It was necessary to perform this experiment in the absence of fatty acid and other systems regenerating ATP. Fig. 3 shows that hydrolysis of added ATP proceeded steadily with time. The addition of 10 units of hexokinase at various time-intervals rapidly removed the ATP so that adenosine-triphosphatase activity ceased. Hexokinase at this concentration was therefore an effective competitor for ATP. When hexokinase was added early there was an actual uptake of inorganic phosphate, due probably to oxidative phosphorylation at the expense of endogenous substrates. The amount of hexokinase used in the experiments to determine P/O ratios was always 20 units.

P/O *ratios as measured by phosphate uptake and manometric estimation of oxygen.* Table 1 shows that the oxidation of butyrate, hexanoate and octanoate, at equivalent concentrations of carbon atoms, was accompanied by ATP formation. The corrected P/O ratios were approximately 2, or above, with butyrate and hexanoate. Octanoate at this concentration (5mM), however, gave varying values (range 0·2–1·5). The oxygen consumption and P/O ratios with octanoate were also extremely variable, and low values for the P/O ratios invariably coincided with low oxygen consumption. It can be seen that with all three fatty acids, on a carbon atom basis, usually 50% or more of the fatty acid metabolized was converted into ketone bodies.

Because of the low P/O ratios at 5mM, the effect of concentration of octanoate was examined further. P/O ratios (1·9) obtained at mM concentration were higher than those (1·1, 0·1) obtained with the same mitochondria at 5mM, and inhibition of oxygen consumption was not apparent at the lower concentration of the fatty acid. The corrected P/O values at the lower concentration were similar to those obtained with butyrate and hexanoate at higher concentrations (Table 1).

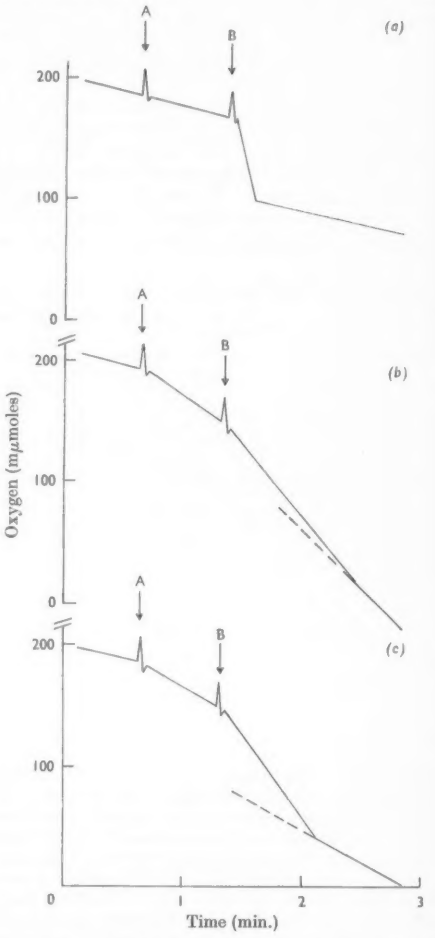


Fig. 4. Polarographic record of oxygen consumption by rat-liver mitochondria. Experimental details are given in the text. (*a*) A, glutamate added (10μmoles), followed by B, ADP (0·4μmole). (*b*) A, octanoate added (0·75μmole), followed by B, ADP (0·4μmole). (*c*) A, octanoate added (0·75μmole) to the medium containing serum albumin (1·0%, w/v), followed by B, ADP (0·4μmole).

Method 2: P/O *ratios as measured by amperometric estimation of oxygen consumption coupled to phosphorylation of ADP*

The estimation of P/O ratios by this short-term method also involves a number of corrections of the observed value. These are considered below.

Apparent presence of adenosine triphosphatase. The results of typical experiments obtained during the oxidation of glutamate and fatty acids are given in Fig. 4. It is assumed that the increase in the rate of oxygen consumption after the addition of ADP is due to the phosphorylation of the latter to ATP and that the subsequent decrease in rate is due to the completion of such phosphorylation. With glutamate (Fig. 4*a*), after the conversion of ADP into ATP, there was little or no increase in oxygen consumption above the pre-ADP rate. In the presence of octanoate (Fig. 4*b*), however, the rate increased. This suggests that the increase in rate of oxygen consumption is due to the establishment of a steady-state concentration of ADP as the result of octanoate-stimulated adenosine-triphosphatase activity with the newly formed ATP as substrate. The magnitude of this increase was less in the presence of serum albumin (Fig. 4*c*).

Such an ADP-regenerating system operating during the phase of ADP phosphorylation to ATP would prolong the oxygen consumption and so lower the observed P/O ratio. It is possible that this apparent adenosine-triphosphatase activity is suppressed during the period of ADP-dependent oxygen consumption, but the lower ratios obtained in the presence of higher concentration of fatty acids are also consistent with the presence of an adenosine triphosphatase with high affinity for ATP. Assuming the presence of such an adenosine-triphosphatase activity during the time of ADP-dependent respiration, its contribution has been corrected for on the basis of one-half the effect observed at the final concentration of ATP obtained (0·27 mM). In most experiments the contribution of this correction to the P/O ratio was less than 10%.

ADP formation from the thiokinase reaction. This correction has been made on the basis of fatty acid utilization/mole of oxygen consumed as determined from the manometric experiments. It has been assumed that, for each fatty acid molecule esterified with coenzyme A, 2 moles of ADP have been formed for each mole of ATP utilized.

P/O *ratios as determined amperometrically.* With each batch of mitochondria the P/O ratios with glutamate were always determined. In 15 experiments, with mitochondria suspended in 0·3 M-sucrose, P/O ratios 3·0 (±0·3) and respiratory control ratios 3·7 (±1·2) were obtained. With mitochondria suspended in the tris–potassium chloride medium of Chappell & Perry (1954), both values were slightly lowered; in five experiments the P/O ratios were 2·8 (±0·1) and respiratory control ratios were 3·3 (±0·6).

Butyrate. Table 2 shows that, with butyrate as substrate, respiratory control was increased in the presence of carnitine both in the presence and absence of serum albumin. This was apparent at all concentrations of butyrate. At the higher concentrations, where respiratory control was low (or absent) and there was no definite change in rate of oxygen consumption to mark the complete phosphorylation of ADP, it was not possible to obtain an accurate estimation of the P/O ratio. The observed and corrected ratios were always highest in the presence of both serum albumin and carnitine and in all cases decreased at the higher concentrations of butyrate. The addition of carnitine usually gave a clear-cut increase in the ratio.

The effect of ADP on the oxygen consumption as related to increase in concentration of the fatty

Table 2. *Effect of concentration of butyrate on oxidative phosphorylation*

Rat-liver mitochondria suspended in sucrose were incubated at 30° in 1·5 ml. of medium in the reaction vessel in which the consumption of oxygen was followed amperometrically, as described in the text. P/O ratio A is the uncorrected ratio as measured directly; P/O ratio B has been corrected for the ADP regenerated during the activation of the fatty acid and for the small increase in apparent adenosine-triphosphatase activity occurring during ADP-dependent oxidation.

	Without serum albumin						With serum albumin					
	Butyrate			Butyrate plus carnitine			Butyrate			Butyrate plus carnitine		
Concn. of butyrate (mM)	Resp. control ratio	P/O ratio		Resp. control ratio	P/O ratio		Resp. control ratio	P/O ratio		Resp. control ratio	P/O ratio	
		A	B		A	B		A	B		A	B
0·1	1·5	1·4	2·0	2·4	1·7	2·3	1·4	1·7	2·3	2·3	1·9	2·5
1·0	1·1	1·4	1·9	2·5	1·7	2·2	1·4	1·1	1·9	2·6	1·6	2·3
10	1·0	1·0	1·5	2·0	1·1	1·6	1·1	1·1	1·6	2·3	1·5	2·2
100	1·0	*		1·3	1·1	1·6	1·0	*		1·2	1·4	2·1

* 'No turnover point': i.e. no change in slope on complete phosphorylation of ADP.

acid is shown in Fig. 5. It is apparent that carnitine markedly stimulated ADP-dependent oxygen consumption; this was more pronounced at lower concentrations of butyrate. A similar result was obtained in the presence of 1·0% serum albumin. Carnitine had no effect in the absence of butyrate and must mediate in some way the oxidation of butyrate and couple it to phosphorylation of ADP.

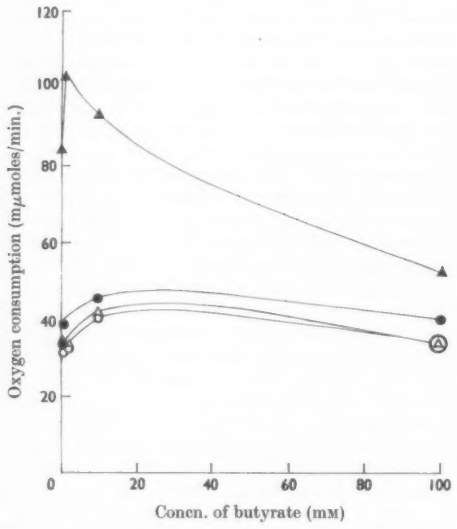


Fig. 5. Effect of butyrate concentration on the rate of oxygen consumption (measured amperometrically) by rat-liver mitochondria. Incubations were performed in the absence of serum albumin; other experimental details are given in the text. In the presence of carnitine: ●, pre-ADP rate; ▲, post-ADP rate. In the absence of carnitine: ○, pre-ADP rate; △, post-ADP rate.

Hexanoate. Table 3 shows that, with hexanoate, the respiratory control ratio and P/O ratio fell with increasing fatty acid concentration. These ratios were greater in the presence of carnitine and/or serum albumin. Figs. 6(*a*) and 6(*b*) show the response in oxygen consumption to increased concentration of hexanoate. It can be seen that the presence of carnitine markedly enhanced the response to ADP and that serum albumin enhanced it still further. The effect of both substances fell off with increase in concentration of the fatty acid.

Octanoate. The general behaviour of octanoate, as shown in Table 4, was similar to that of butyrate and hexanoate, up to a concentration of 1mM. Above this concentration, except in the presence of 1·0% serum albumin plus carnitine, the polarographic traces showed a fall in rate of oxygen consumption with time and respiratory control ratios could not be determined. Because there was no indication of the point of complete phosphorylation of ADP, P/O ratios at concentrations above 1 mM could only be determined when both serum albumin and carnitine were present together in the medium. Serum albumin had a more marked coupling effect than carnitine. As with the two lower fatty acids, higher P/O ratios were obtained in the presence of serum albumin and carnitine at the lower fatty acid concentrations. The corrected ratio of 2·6 at octanoate concentration 0·1mM indicates efficient ATP formation with this fatty acid.

Figs. 7(*a*) and 7(*b*) show that at low concentrations of octanoate there was the usual marked effect of ADP and that this was further increased in the presence of carnitine and serum albumin. Increase in the concentration of octanoate up to 3mM in the absence of added ADP stimulated oxygen consumption. The addition of serum albumin shifted the curves towards a higher concentration of the fatty acid before the maximum was reached. At concentrations above 3–5mM, the actual concentration depending on the presence or

Table 3. *Effect of concentration of hexanoate on oxidative phosphorylation*

Rat-liver mitochondria suspended in sucrose were incubated as for Table 2.

	Without serum albumin						With serum albumin					
	Hexanoate			Hexanoate plus carnitine			Hexanoate			Hexanoate plus carnitine		
Concn. of hexanoate (mM)	Resp. control ratio	P/O ratio		Resp. control ratio	P/O ratio		Resp. control ratio	P/O ratio		Resp. control ratio	P/O ratio	
		A	B		A	B		A	B		A	B
1·0	1·6	1·5	2·0	2·3	1·7	2·0	2·2	1·7	2·1	3·6	1·9	2·3
10	1·3	0·9	1·3	1·4	1·3	1·7	1·5	1·4	1·8	1·8	1·5	1·9
20	1·1	0·6	1·0	1·2	0·7	1·1	1·3	1·2	1·5	1·4	1·3	1·7

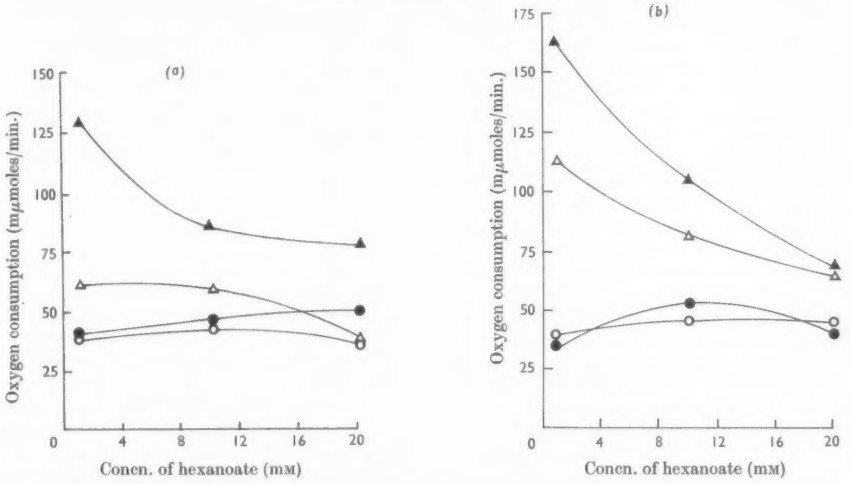


Fig. 6. Effect of hexanoate concentration on the rate of oxygen consumption (measured amperometrically) by rat-liver mitochondria; other experimental details are given in the text. Incubations were performed in the absence (*a*) and presence (*b*) of serum albumin (1%, w/v). In the presence of carnitine: ●, pre-ADP rate; ▲, post-ADP rate. In the absence of carnitine: ○, pre-ADP rate; △, post-ADP rate.

Table 4. *Effect of concentration of octanoate on oxidative phosphorylation*

Rat-liver mitochondria suspended in sucrose were incubated as for Table 2. Where no respiratory control ratio is given, the fatty acid caused a progressive inhibition of oxygen consumption.

	Without serum albumin						With serum albumin					
	Octanoate			Octanoate plus carnitine			Octanoate			Octanoate plus carnitine		
Concn. of octanoate (mM)	Resp. control ratio	P/O ratio A	P/O ratio B	Resp. control ratio	P/O ratio A	P/O ratio B	Resp. control ratio	P/O ratio A	P/O ratio B	Resp. control ratio	P/O ratio A	P/O ratio B
0·1	1·8	1·7	2·2	1·6	1·6	2·1	1·8	1·9	2·3	2·4	2·2	2·6
0·5	1·5	1·5	1·9	1·5	1·4	1·8	2·2	2·0	2·3	2·7	2·2	2·5
1·0	1·2	1·0	1·3	1·3	1·1	1·4	1·8	1·9	2·2	2·2	2·0	2·3
2·0	—	*		1·1	*		1·4	*		1·8	1·6	1·9
3·0	—	*		—	*		1·0	*		1·5	1·3	1·6
5·0	—	*		—	*		—	*		1·0	*	

* 'No turnover point': i.e. no change in slope on complete phosphorylation of ADP.

absence of carnitine or serum albumin, octanoate became inhibitory and showed no response to ADP.

Fig. 8 shows the effect of increasing the concentration of serum albumin on the rate of oxidation of octanoate and the resulting P/O ratios. At this concentration (3·5mM) of octanoate, in the absence of serum albumin, oxygen consumption is not tightly coupled with phosphorylation of ADP. It can be seen that the rate of oxygen consumption in the presence of ADP rose steeply up to 1·0% serum albumin and thereafter quite slowly. With increasing concentration of serum albumin the P/O ratio also rose and was at a maximum at about 3·0% serum albumin. The rate of oxygen consumption before the addition of ADP is also given for comparison.

Time-course of the response to carnitine. Fig. 9 shows that when butyrate was oxidized in the

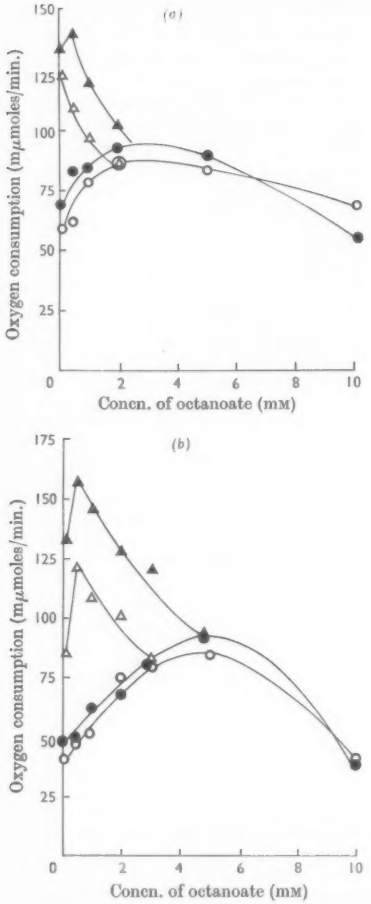


Fig. 7. Effect of octanoate concentration on the rate of oxygen concentration (measured amperometrically) by rat-liver mitochondria; other experimental details are given in the text. Incubations were performed in the absence (*a*) and presence (*b*) of serum albumin (1%, w/v). In the presence of carnitine: ●, pre-ADP rate; ▲, post-ADP rate. In the absence of carnitine: ○, pre-ADP rate; △, post-ADP rate.

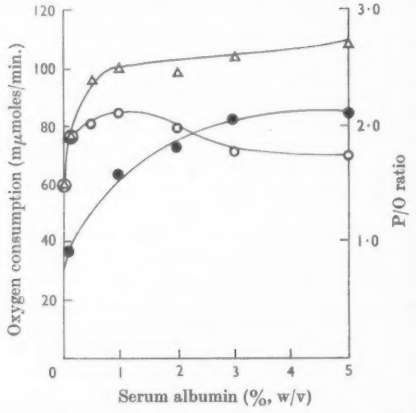


Fig. 8. Effect of serum albumin on oxidative phosphorylation and the rate of oxygen consumption (measured amperometrically) by rat-liver mitochondria in the presence of octanoate (3·5mM); other experimental details are given in the text. ○, Pre-ADP rate; △, post-ADP rate; ●, P/O ratio (corrected).

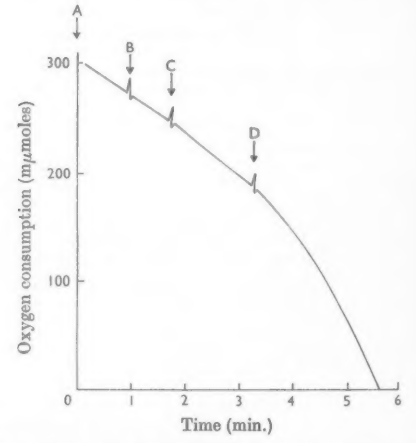


Fig. 9. Polarographic record of oxygen consumption by rat-liver mitochondria in the presence of butyrate. Incubating medium contained serum albumin (1%, w/v) and other constituents as given in the text. Additions were: A, mitochondria (0·1ml.); B, butyrate (15μmoles); C, ADP (0·8μmole); D, carnitine (1·5μmoles).

presence of ADP, the effect of carnitine on the respiration rate was not immediate but continued for up to 1·5min.

Comparison of the two suspending media. P/O ratios estimated by the manometric and amperometric procedures were carried out with different suspending media. In the manometric experiments this was done so that ketone bodies could be estimated without interference from sucrose. With octanoate (0·5 and 1·0mM) as substrate, and by using the amperometric method, there were detect-

able differences between mitochondria finally suspended in 0·3M-sucrose and in the tris–potassium chloride medium (Chappell & Perry, 1954), but these were quite small. The addition of carnitine and serum albumin, separately and together, increased the P/O ratio more markedly with the sucrose medium. Similar results were obtained with butyrate and hexanoate.

DISCUSSION

It is apparent from the results presented above that fatty acid oxidation is accompanied by ATP formation, giving P/O ratios similar to those expected for the oxidation of $FADH_2$ plus $NADH_2$. This is only the case when the concentration of the fatty acid is very low or when the reaction mixture contains sufficient serum albumin to overcome the deleterious effects of the higher concentrations of the fatty acids. Increase in the concentration of the short-chain fatty acids used causes uncoupling of oxidative phosphorylation, loss of respiratory control and, finally, inhibition of the rate of oxygen consumption. The concentration at which such effects begin is inversely related to the chain length of the fatty acid. It has been shown previously that, of the saturated series, tetradecanoic acid is the most potent uncoupler of oxidative phosphorylation, inhibitor of mitochondrial respiration (Björntorp *et al.* 1964) and of tissue respiration (Enser, 1964), and activator of adenosine triphosphatase (Pressman & Lardy, 1956). Because of the high surface activity of this fatty acid, Björntorp *et al.* (1964) have suggested the possibility of a correlation between uncoupling and surface activity.

It is possible that the fatty acids inhibit respiration by acting directly on the enzymes associated with their oxidation or on the enzymes of the electron-transport system. Succinate oxidase is inhibited in the presence of sodium oleate and other unsaturated fatty acids in such a manner (Edwards & Ball, 1954; Nakamura *et al.* 1959).

The stimulation of fatty acid oxidation by serum albumin is possibly related to the binding of fatty acids to the protein molecule. Teresi & Luck (1952) have shown that both hexanoate and octanoate can be bound to the protein in the ratio of 32moles/mole of albumin. Removal by binding is a satisfactory explanation for the stimulation of respiration which occurs when the concentration of fatty acid is sufficient to cause respiratory inhibition in the absence of serum albumin. But the stimulation of butyrate metabolism at 10mM (Fig. 1), where there is little change in the P/O ratio on addition of serum albumin (Table 2), would require a different explanation. Catabolism of serum albumin by rat-liver mitochondrial preparations has been shown to occur (Penn, 1960) and the metabolism of glutamate and perhaps other amino acids is known to stimulate the oxidation of fatty acids (Hird, Symons & Weidemann, 1966). The effect of serum albumin in the manometric experiments, but perhaps not in the short-term amperometric determinations, is explicable in these terms.

The present work shows that the addition of carnitine to the incubation mixture increases the rate of oxidation of the short-chain fatty acids, but except with butyrate this has not previously been observed (Fritz, Kaplan & Yue, 1962). We have no explanation for the different results. The results found here are consistent with the idea that carnitine facilitates the transport of fatty acids (including those of short-chain length) to the site of oxidation within the mitochondria (Fritz, 1963). The present work shows that carnitine can also restore coupling between oxidation and phosphorylation. Similar observations with coenzyme A have been made by McMurray & Lardy (1958) and Björntorp *et al.* (1964). Björntorp *et al.* (1964) and Lehninger & Remmert (1959) explain the effect in terms of the local concentration of deleterious fatty acid being lowered by acylation. Carnitine may also act in this way.

It is possible that the increased P/O ratio observed in the presence of carnitine is only apparent, and is simply related to the short duration of the experiments by the amperometric method. An accumulation of the fatty acid as acyl-carnitine at the expense of endogenous ATP could lead to an overestimate of the P/O ratio value due to the application of a now spurious correction for ATP utilization in the activation reaction. However, as the total effect of carnitine on the system includes stimulation of oxygen consumption and increased respiratory control as well as increased P/O ratios, these speculations are inadequate as a full explanation.

The authors acknowledge financial support from the Commonwealth Bank Rural Credits Fund and the Australian Wool Research Committee.

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The Effect of Hexokinase and Tricarboxylic Acid-Cycle Intermediates on Fatty Acid Oxidation and Formation of Ketone Bodies by Rat-Liver Mitochondria

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(*Received* 31 *August* 1965)

1. The oxidation of butyrate, hexanoate and octanoate by rat-liver mitochondria suspended in a tris–potassium chloride medium in the presence of malate and serum albumin has been investigated. 2. The oxidation of butyrate to acetoacetate was markedly decreased by the addition of a system competitive for ATP (hexokinase–glucose). 3. Serum albumin or tricarboxylic acid-cycle intermediates prevented the inhibition by hexokinase and in their presence a greater proportion of the oxygen consumption was contributed by the tricarboxylic acid cycle. The results suggest that the energy supply for fatty acid activation is either compartmentalized in a spatial or kinetic sense or there exists a special activating mechanism not involving ATP. 4. Malate and other tricarboxylic acid-cycle intermediates caused substantial reduction (to β-hydroxybutyrate) of the acetoacetate formed during the oxidation of butyrate, hexanoate and octanoate.

The oxidation of fatty acids by mitochondria is known to be stimulated by the addition of catalytic amounts of malate and other tricarboxylic acid-cycle intermediates (Knox, Noyce & Auerbach, 1948; Kennedy & Lehninger, 1949).

The effect of tricarboxylic acid-cycle intermediates on such systems has often been interpreted as a need for oxaloacetate, to accept and initiate the oxidation of acetyl groups. It has also been suggested that the concurrent metabolism of added intermediates provides energy in some form for the initial activation of the fatty acid (Knox *et al.* 1948; Cross, Taggart, Covo & Green, 1949).

The present paper reports the result of an investigation on the effect of tricarboxylic acid-cycle intermediates and serum albumin as stimulators of fatty acid oxidation by mitochondria and of hexokinase as a competitor for available adenosine triphosphate.

EXPERIMENTAL

Materials

Crystalline hexokinase (170000 units/g. at 38°; for definition of one unit see the preceding paper) and ATP were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and crystalline bovine serum albumin from the Commonwealth Serum Laboratories, Melbourne, Australia. The hexokinase and serum albumin were dialysed at 2° against water before use and the activity of the hexokinase was estimated as described by Hird & Weidemann (1966). The fatty acids were distilled and converted into their potassium salts. All other reagents used were of analytical grade.

Methods

Rat-liver mitochondria were prepared by the method described by Hird & Symons (1962) and were finally suspended in the tris–KCl medium of Chappell & Perry (1954). The final mitochondrial suspension in 4ml. was derived from 10g. of liver (fresh wt.). Incubation was carried out at 38° in Warburg flasks in the medium described by Hird & Weidemann (1966), in a final volume 3·0ml., 0·3ml. of the mitochondrial suspension being used. Glucose and hexokinase, when added, were tipped from the side arm at the beginning of an incubation period of 30–50min. At the end of the experiment, protein was removed from the medium by the method of Weichselbaum & Somogyi (1941). The fatty acids remaining were estimated by steam-distillation and titration under CO_2-free conditions (Pennington, 1952) and ketone bodies were estimated as described by Hird & Weidemann (1964).

RESULTS

Competition between hexokinase and thiokinase for ATP

Fig. 1 shows the effect of increasing the amount of hexokinase on the oxidation of butyrate. As the hexokinase was increased, oxygen consumption steadily decreased and there was a corresponding decrease in disappearance of fatty acid and production of ketone bodies. The close parallel between

fatty acid disappearance and ketone-body production suggests that under these conditions there was little oxidation through the tricarboxylic acid cycle. It was a general feature of these experiments that fatty acid oxidation could proceed without the necessity of an ADP-regenerating system (hexokinase–glucose). This could occur if a supply of ADP was made available by a mitochondrial 'adenosine triphosphatase' stimulated by the presence of the fatty acid (see also Hird & Weidemann, 1966). Because fatty acid oxidation was almost completely abolished at 10 units of hexokinase and above, the glucose–hexokinase system apparently prevented esterification of the fatty acid with coenzyme A by removal of ATP.

Fig. 2 shows the same experiment performed in the presence of malate (mM), which is known to have a marked effect on the oxidation of butyrate. In these experiments, oxygen consumption in the absence of hexokinase was much greater and was stimulated by the addition of up to 2 units of the enzyme before flattening off at the higher concentrations. In contrast with the system without malate, hexokinase had little inhibitory effect on the rate of oxygen consumption, even at a concentration of 50 units. It is apparent that in the presence of malate the system activating fatty acids (thiokinase reaction) represents the preferred pathway of ATP utilization, as the glucose–hexokinase seems unable to compete effectively. Fig. 2 also shows that ketone-body production fell off with increasing hexokinase, but it too was not abolished. As the hexokinase amount was increased from 0 to 2 units, the curves representing oxygen consump-

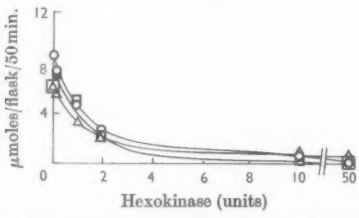

Fig. 1. Effect of hexokinase concentration on the oxidation of butyrate by rat-liver mitochondria. Experimental details are given in the text. Incubation medium contained butyrate (10 mM). ○, Oxygen consumption; △, ketone-body production; □, butyrate disappearance.

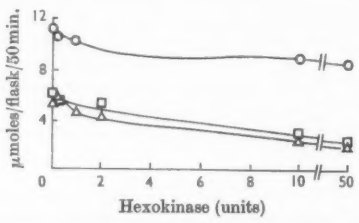

Fig. 3. Effect of serum albumin on the oxidation of butyrate by rat-liver mitochondria. Experimental details are given in the text. Incubation mixture contained butyrate (10 mM) and serum albumin (1%, w/v). ○, Oxygen consumption; △, ketone-body production; □, butyrate disappearance.

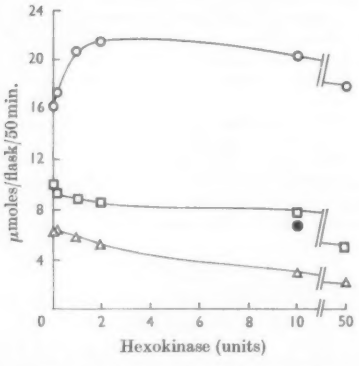

Fig. 2. Effect of malate on the oxidation of butyrate by rat-liver mitochondria. Experimental details are given in the text. Incubation mixture contained butyrate (10 mM) and malate (1 mM). ○, Oxygen consumption; △, ketone-body production; □, butyrate disappearance; ●, oxygen consumption in the presence of malate alone.

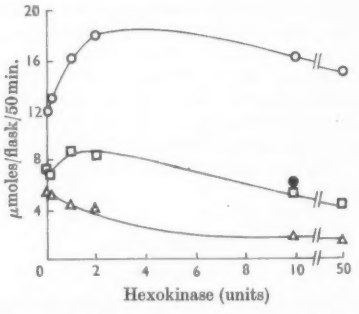

Fig. 4. Effect of malate and serum albumin on the oxidation of butyrate by rat-liver mitochondria. Experimental details are given in the text. Incubation mixture contained butyrate (10 mM), malate (1 mM) and serum albumin (1%, w/v). ○, Oxygen consumption; △, ketone-body production; □, butyrate disappearance; ●, oxygen consumption in the presence of malate and serum albumin.

tion and ketone-body production diverged, indicating that a progressively greater proportion of the fatty acid was metabolized through the tricarboxylic acid cycle. An overall effect was that in the presence of malate a much greater proportion of the total oxygen consumption was contributed by the tricarboxylic acid cycle.

Fig. 3 shows the effect of increasing the amount of hexokinase when serum albumin (1·0%, w/v) was also added to the system. Comparison of Figs. 1 and 3 shows that, apart from a lack of stimulation of oxygen consumption at 2 units of hexokinase, serum albumin simulated at a lower concentration the effect of malate. When both malate and serum albumin were present in the system (Fig. 4) there was no marked additive effect but a small stimulation of fatty acid removal by low concentrations of hexokinase occurred.

To test the possibility that serum albumin stimulated fatty acid oxidation by direct inhibition of hexokinase, the latter was assayed in the presence of concentrations of up to 5% (w/v) of this protein. No inhibition of hexokinase activity was observed.

Effect of tricarboxylic acid-cycle intermediates on the metabolism of fatty acids

Table 1 shows the effect of malate and related intermediates on the oxidation of butyrate in the presence and absence of serum albumin. In the presence and absence of malate the amount of hexokinase added (2 units) caused either a marked stimulation or inhibition of butyrate oxidation. Succinate, glutamate, α-oxoglutarate and citrate as well as malate all markedly stimulated the oxidation of butyrate as measured by oxygen consumption. The oxidation of butyrate to ketone bodies was also increased, but the percentage stimulation was much less than that for oxygen consumption. The stimulation of butyrate oxidation by malate and other tricarboxylic acid-cycle intermediates therefore leads to preferential metabolism of the fatty acid through the tricarboxylic acid cycle as the ratio of moles of ketone bodies formed/moles of oxygen consumed falls (Table 1). A further effect of the addition of such intermediates was the high proportion of the total ketone bodies as β-hydroxybutyrate. The presence of serum albumin in the reaction mixture led to an increase in the oxygen consumption and this was usually accompanied by an increase in ketone-body production.

In Table 2 the oxidation of hexanoate and octanoate is shown in relation to that of butyrate. The oxidation of these fatty acids was also markedly stimulated by malate in the presence of serum albumin. In each case ketone-body production was stimulated and there was considerable reduction of acetoacetate to β-hydroxybutyrate. With malate and the longer-chain fatty acids, ketone-body production was stimulated to a greater extent than oxygen consumption.

Table 1. *Stimulation of oxidation of butyrate by malate and metabolically related compounds*

Rat-liver mitochondria suspended in the tris–KCl medium of Chappell & Perry (1954) were incubated for 30 min. at 38° in 3·0 ml. of medium containing butyrate (10 mM). Hexokinase (2 units) was added to each flask and serum albumin (0·5%, w/v) was added where indicated. Results are expressed in μmoles/0·3 ml. of mitochondrial suspension/30 min.

Expt. no.	Butyrate (10 mM) plus additions (0·3 mM)	Serum albumin (0·5%)	Oxygen consumption (μmoles)	Ketone bodies formed (μmoles)	Ketone bodies formed β-Hydroxybutyrate (%)	Ratio moles of ketone bodies formed / moles of O_2 consumed
1	None	−	1·9	1·5	0	0·80
		+	6·0	2·1	5	0·35
	Malate	−	11·7	2·2	41	0·19
		+	13·5	2·3	48	0·17
	Succinate	−	11·6	2·4	47	0·21
		+	13·8	3·3	63	0·24
	Glutamate	−	9·9	3·1	26	0·32
		+	12·2	3·4	33	0·28
2	None	−	2·6	2·0	10	0·78
		+	6·9	2·7	16	0·39
	Malate	−	13·0	2·8	43	0·22
		+	14·5	3·0	58	0·21
	α-Oxoglutarate	−	5·3	3·0	28	0·57
		+	9·1	3·1	26	0·35
	Citrate	−	15·5	3·6	54	0·23
		+	15·9	3·6	65	0·22

Table 2. *Stimulation of oxidation of butyrate, hexanoate and octanoate by malate*

Rat-liver mitochondria suspended in the tris–KCl medium of Chappell & Perry (1954) were incubated as described in Table 1. All flasks contained hexokinase (2 units) and serum albumin (0·5%, w/v). Results are expressed in μmoles/0·3 ml. of mitochondrial suspension/30 min.

Expt. no.	Additions	Oxygen consumption (μmoles)	Ketone bodies formed (μmoles)	Ketone bodies formed β-Hydroxybutyrate (%)	Ratio moles of ketone bodies formed / moles of O_2 consumed
1	Butyrate (10 mM) plus:				
	—	8·4	4·0	10	0·48
	malate (0·3 mM)	16·5	6·4	46	0·39
2	Hexanoate (7·5 mM) plus:				
	—	6·4	2·3	0	0·35
	malate (0·3 mM)	10·4	5·4	65	0·52
3	Octanoate (5 mM) plus:				
	—	9·0	2·9	0	0·33
	malate (0·3 mM)	11·0	5·0	40	0·45

DISCUSSION

Stimulation of fatty acid oxidation. Under certain experimental conditions, the oxidation of fatty acids may be limited by the availability of ATP, especially when reactions competing for ATP are present in the system. Hexokinase has been shown to compete successfully with the activating system for fatty acid oxidation (thiokinase reaction) and in high concentration can completely inhibit the metabolism of the fatty acid. The addition of a small amount of malate prevents this inhibition. Presumably malate, by being metabolized as such and/or by acting as a source of oxaloacetate, stimulates the tricarboxylic acid cycle and provides ATP, or some equivalent energy source, at an increased rate, or in a form which is available for the activation reaction but which is unavailable to the extramitochondrial hexokinase.

The stimulation of fatty acid oxidation by tricarboxylic acid-cycle intermediates has been shown by Cross *et al.* (1949) to be inhibited by uncoupling agents. It is known, also, that the substitution of $NADH_2$ for such intermediates promotes the activation of octanoate and its subsequent oxidation to acetoacetate (Kennedy & Lehninger, 1951). In each case the increased metabolism of the fatty acid can be attributed to an increased supply of ATP for the activation of the fatty acid. In the presence of malonate the addition of intermediates of the tricarboxylic acid cycle stimulates the conversion of fatty acid into acetoacetate, the stimulation in this case being apparently unrelated to the availability of oxaloacetate (Cheldelin & Beinert, 1952).

A simple explanation of the accumulated facts is that, in the presence of malate and serum albumin, newly formed ATP is either (*a*) compartmentalized, i.e. it is available to the activating enzymes and substantially unavailable to hexokinase and similar enzymes utilizing ATP which are exterior to the mitochondrion, or (*b*) in the presence of malate it is produced at a faster rate with more being available for each competing reaction. The existence of a barrier to exogenous ATP at the level of the mitochondrial membrane is suggested by the work of Judah & Rees (1953). It is also possible (Bode & Klingenberg, 1964) that intermediates other than ATP, i.e. intermediates of high group transfer potential produced by the cytochrome system during the oxidation of tricarboxylic acid intermediates, or perhaps other nucleotide triphosphates, may serve for the activation of fatty acids. In this connexion a GTP-specific thiokinase has recently been identified in ox-liver mitochondria (Rossi & Gibson, 1964). There is also the possibility of thiophorase reactions leading to formation of fatty acid-CoA esters, i.e. derived from acetyl-CoA and succinyl-CoA.

The stimulation of oxidation by serum albumin is not readily explicable. Perhaps by stabilizing mitochondrial permeability in some way it raises the internal energy supply or restricts the availability of ATP to the glucose–hexokinase system. There is the possibility, however, that it may, by its catabolism (Penn, 1960), contribute precursors of tricarboxylic acid-cycle intermediates by metabolism of related amino acids.

Direction of fatty acid oxidation. The preferential direction of acetyl-CoA into the tricarboxylic acid cycle by malate and other intermediates is a separate effect from that which causes the overall increase in metabolism of the fatty acid. The oxidation of fatty acids to acetoacetate does not require

the presence of endogenous oxaloacetate or its precursors (Lehninger, 1946; Cheldelin & Beinert, 1952), but the addition of these, in the absence of malonate, does, however, lower the proportion of fatty acid converted into acetoacetate (Lehninger, 1946). This effect has also been shown with carnitine esters of fatty acids when succinate is added to the system (Bremer, 1962). The most likely explanation is that the provision of oxaloacetate initiates metabolism through the tricarboxylic acid cycle. There is, however, the possibility that metabolic direction in this case is mediated by the effect of saturation on the kinetics of the two pathways.

Formation of ketone bodies. Substantial reduction of the acetoacetate formed during fatty acid oxidation has been shown to occur when malate or some other tricarboxylic acid-cycle intermediate is added to the medium. The reduction of added acetoacetate by succinate (Ernster, 1962; Kulka, Krebs & Eggleston, 1961; Azzone, Ernster & Weinbach, 1963), and of newly synthesized acetoacetate from butyrate, by rumen epithelium in the presence of glucose has been previously established (Hird & Symons, 1959). From the present results it is apparent that the power to reduce acetoacetate can come from a number of sources, but the reduced coenzymes formed during the oxidation of fatty acids are not by themselves very effective sources of hydrogen.

The authors acknowledge financial support from the Commonwealth Bank Rural Credits Development Fund and the Australian Wool Research Committee.

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Transport and Metabolism of Fatty Acids by Isolated Rumen Epithelium

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(*Received* 31 *August* 1965)

1. The metabolism of even-numbered saturated (acetic acid to stearic acid) and unsaturated (oleic acid and linolenic acid) fatty acids by diaphragms of isolated rumen epithelium has been investigated. 2. When fatty acids are presented to the papillae surface, ketone bodies are released from the opposite (muscle) side of the tissue. 3. When the concentration of octanoate or decanoate is increased to a critical value, which varies inversely with the chain length of the fatty acid, the respiration of the tissue is inhibited and ketone body synthesis is diminished. Under these conditions unmetabolized fatty acid crosses the tissue down a concentration gradient. 4. The inhibitions by octanoate and decanoate are more marked when the fatty acid is presented to both surfaces of the rumen epithelium. 5. During the oxidation of octanoate and decanoate at non-inhibitory concentrations, small quantities of shorter chain fatty acids, including acetate, are produced.

The rumen epithelium of the sheep is in contact with short- and long-chain fatty acids. The former are the products of microbial fermentation (see review by Warner, 1964) and the latter are derived in much smaller amount mainly from the galactosylglycerides of fodder plants (Weenink, 1959; Garton, 1960; van der Veen & Olcott, 1964). These are hydrolysed and the free fatty acids substantially hydrogenated by the rumen flora (Shorland, Weenink & Johns, 1955; Garton, Lough & Vioque, 1961). Acetate, propionate and butyrate are metabolized, the last-named to ketone bodies, by the rumen epithelium both *in vivo* and *in vitro* (Pennington, 1952). When presented to the papillae surface of such tissue *in vitro*, butyrate gives rise to a preferential release of ketone bodies at the opposite (muscle) surface of the tissue (Hird & Weidemann, 1964*a*). Hexanoate (Plaut & Smith, 1951), octanoate and decanoate (Schultz, Smith & Lardy, 1949) have been shown to give rise to increased blood ketone bodies when administered orally to goats. In experiments with isolated rumen epithelium of the sheep, Jackson, Taylor, Hatcher & Carter (1964) have shown that palmitic acid, stearic acid, oleic acid and linoleic acid are also precursors of ketone bodies.

In the present paper the metabolism and transport of fatty acids by isolated sheets of rumen epithelium of the sheep have been investigated.

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EXPERIMENTAL

Materials

Sodium butyrate, hexanoate, octanoate and decanoate were prepared from their redistilled acids (British Drug Houses Ltd., Poole, Dorset). Lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid and linoleic acid were purchased from the Hormel Institute, Austin, Minn., U.S.A., and were converted into the sodium salts before use. Crystalline bovine serum albumin was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. The other reagents used were of analytical grade.

Methods

Experimental procedure. Preparation of the rumen epithelium tissue (from which the accompanying muscle layers had been removed) and its incubation at 38° as a diaphragm supported by two Perspex plates was carried out as described by Hird & Weidemann (1964*a*), except that both compartments of the apparatus were of the same size (diam. 6·8 cm., depth 0·7 cm.) with a capacity of approx. 25 ml. Oxygen was passed through channels drilled into the Perspex plates and the stream of gas bubbles served to oxygenate and to mix the contents. The use of four units allowed four separate comparisons to be made on tissue from the same animal.

The effect of concentration of octanoate and decanoate on the consumption of O_2 of rumen epithelium was followed in a Warburg apparatus at 38° over a period of 2 hr. Rumen

epithelium bearing small papillae was obtained from the dorsal sac and the preparation of the tissue and its incubation in the presence of substrates were performed as described by Hird & Symons (1959). Results are expressed as μmoles/50mg. dry wt./2hr.

Estimation of fatty acids. Estimations were made in duplicate. Butyrate and acetate were estimated by steam-distillation and titration as described by Pennington (1952).

With hexanoate, octanoate and decanoate the estimations were performed by gas–liquid chromatography. The method used was as follows.

Samples of the incubation mixtures (2·0 or 5·0ml.) were adjusted approximately to pH11 or greater by addition of NaOH (2M) and a known amount of the sodium salt of a suitable fatty acid (Table 1) was added to each sample to provide an internal standard. The solution was then evaporated to dryness at 95–100° in a stream of N_2. The residue was acidified with saturated $NaHSO_4$ and diethyl ether (4ml.) was added, followed by anhydrous Na_2SO_4 (1·5g.). The mixture was ground and mixed and the ethereal solution transferred to a 15ml. tapered centrifuge tube. A second extraction of the Na_2SO_4 was made with diethyl ether (2ml.) and the combined extracts were concentrated to about 0·3ml. in a water bath at 45–50°.

The free acids were analysed in a gas chromatograph (model 300; F & M Scientific Corp., Acton, London, W. 3). The detector was operated with a filament current of 165mA and at a temperature of 185°. The stainless-steel column (122cm.×4mm.) was packed with diethylene glycol–succinate (5%, w/w) and orthophosphoric acid (0·7%, w/w) adsorbed on a fluoroethylene polymer (Haloport F; F & M Scientific Corp.). Samples (up to 0·1ml.) of the ethereal solutions were applied directly to a plug of glass wool at the top of the column to avoid adsorption of fatty acids in the injection port. After starting the carrier gas, the temperature was maintained at 70° for 5min. to remove ether and any residual water from the sample. The temperature was then raised rapidly (about 80°/min.) to an operating temperature within the experimental range 125–145° (Table 1). The flow-rate and operating temperature were selected to give a retention time of 20–30min. for the slowest component.

Estimation of ketone bodies. This was done by the method described by Hird & Symons (1959) as modified by Hird & Weidemann (1964*b*).

Calculations. The results in Tables 2 and 3 give the distribution of ketone bodies on both sides of the tissue after 2-hr. incubations. The total number of μmoles of fatty acid appearing on the opposite side of the tissue is shown as fatty acid 'transported'. The loss or gain of fatty acid is given for comparison; this figure represents the difference between the number of μmoles of fatty acid added initially and the recovery of fatty acid on both sides of the tissue at the end of the experiment. It is assumed that loss of fatty acid in this context includes some retention of unmetabolized fatty acid in the tissue, as well as loss by metabolism to ketone bodies, CO_2 and water. The extent of retention in the tissue has not been estimated.

Table 1. *Conditions for gas chromatography of fatty acids*

Other experimental details are given in the text.

Substrate	Internal standard	Carrier gas-flow rate (ml./min.)	Operating temp.
Hexanoate	Valerate	25	125°
Octanoate	Nonanoate	50	135
Decanoate	Nonanoate	50	145

RESULTS

Transport experiments with 10mM *solutions.* Table 2 shows the results of experiments where fatty acids were applied to the papillae surface of isolated rumen epithelium. In each case ketone bodies appeared on the muscle side of the tissue and very little appeared on the papillae side. With acetate as substrate, total ketone-body production was only slightly higher than with no added substrate and there was no net disappearance of acetate from the system. A small amount of acetate

Table 2. *Transport and metabolism of fatty acids by isolated rumen epithelium*

Substrates were added only to the papillae surface of isolated rumen epithelium (bearing small papillae). Incubation at 38° for 2hr. was as given in the text. Results are calculated as μmoles/diaphragm/2hr. Standard deviations are given in parentheses.

Additions (10mM) to the papillae side	No. of expts.	Total ketone bodies (μmoles)		Fatty acid (μmoles)	
		Muscle side	Papillae side	Transported to muscle side	Loss or gain
None	2	3 (±1)	0·4 (±0·1)	—	+3 (±2)
Acetate	3	4 (±0·5)	0·4 (±0·3)	11 (±2)	+1 (±3)
Butyrate	2	24 (±3)	4 (±0)	8 (±2)	−40 (±4)
Hexanoate	4	33 (±16)*	4 (±2)	9 (±4)	−38 (±12)
Octanoate	3	17 (±3)	3 (±2)	25 (±3)	−18 (±5)
Decanoate	4	3 (±1)	0·6 (±0·4)	30 (±1)	−15 (±4)

* The large variation is due to one low figure (9μmoles).

Table 3. *Transport and metabolism of fatty acids by isolated rumen epithelium*

Substrates were added only to the muscle surface of isolated rumen epithelium; other conditions were as described for Table 2.

Additions (10 mM) to the muscle side	No. of expts.	Total ketone bodies (μmoles)		Fatty acid (μmoles)	
		Muscle side	Papillae side	Transported to papillae side	Loss or gain
None	2	3 (±1)	0·4 (±0·1)	—	+3 (±2)
Acetate	4	5 (±0)	0·7 (±0·4)	12 (±6)	−23 (±6)
Butyrate	3	52 (±9)	3 (±2)	5 (±1)	−63 (±8)
Hexanoate	4	44 (±18)*	4 (±2)	21 (±6)	−51 (±12)
Octanoate	1	18	2	37	−17
Decanoate	2	3 (±2)	0·8 (±0·2)	37 (±1)	−20 (±1)

* The large variation is due to one low figure (14 μmoles).

was transported to the muscle side under these conditions.

The distribution of ketone bodies across the diaphragm and the amount of intact fatty acid appearing on the muscle side when hexanoate was the substrate were similar to results previously obtained with butyrate (Hird & Weidemann, 1964*a*). However, as the chain length of the fatty acid increased to octanoate and decanoate, the amount of unmetabolized fatty acid transported was considerable and was greater than the total number of moles of ketone bodies formed. With decanoate at this concentration (10 mM), ketone-body production was very small, but with hexanoate and octanoate, the number of moles of fatty acid disappearing from the system was similar to the number of moles of ketone bodies synthesized. The results suggest that with butyrate, hexanoate and octanoate substantial metabolism of acetyl groups occurs to products other than ketone bodies. With decanoate there was a much greater loss of fatty acid not accounted for by ketone-body production. It is assumed from the later results, which show a low rate of respiration at this concentration of decanoate, that the fatty acid substantially remained in the tissue at the end of the experiment.

The results of related experiments, where the fatty acids were applied to the muscle surface of the tissue, are given in Table 3. It is apparent that the marked distribution of ketone bodies in favour of the muscle side was similar with each fatty acid and that once again decanoate was metabolized to only a small extent. The results also indicate that fatty acid moved through the tissue to the papillae side. This was again greatest with octanoate and decanoate and on a mole to mole basis exceeded total ketone-body production. As before, there was a substantial disappearance of fatty acid from the system, which was mostly related to ketone-body formation (on a molar basis) except with acetate and decanoate. The greater metabolism of acetate by the muscle surface is probably a reflection of increased access of acetate to metabolic sites through greater permeability of this surface. With butyrate and hexanoate, but not with octanoate and decanoate, application to the muscle surface also led to a greater conversion of the fatty acid carbon atoms into ketone bodies. The large disappearance of decanoate in relation to the small

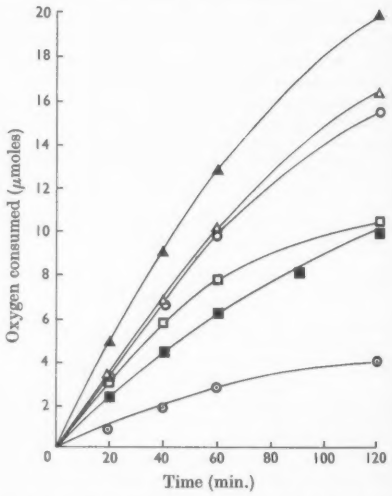

Fig. 1. Effect of octanoate concentration on the rate of oxygen consumption by isolated rumen epithelium. Incubations were carried out in Warburg flasks at 38° for 2 hr. as described in the text. Results are expressed in μmoles/50 mg. dry wt. of tissue. ■, No added substrate; ▲, butyrate (10 mM). Concentrations of octanoate: ○, 1 mM; △, 2 mM; □, 10 mM; ⊙, 20 mM.

total ketone-body formation is probably due again to retention of unmetabolized fatty acid in the tissue.

Effect of concentration of octanoate and decanoate on fatty acid transport and metabolism by rumen epithelium

Oxygen consumption. The results given in Tables 2 and 3 for decanoate (10mM) may indicate an inhibition of tissue respiration at this concentration of the fatty acid. A similar inhibition has been shown for adipose tissue in the presence of fatty acid by Hagen & Ball (1961). Accordingly, the influence of decanoate and octanoate on tissue respiration has been investigated over a range of concentrations.

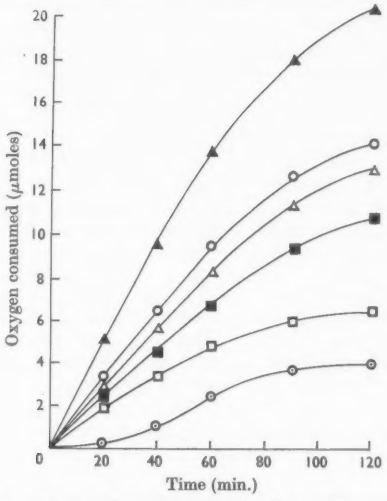

Fig. 2. Effect of decanoate concentration on the rate of oxygen consumption by isolated rumen epithelium. Experimental details were as for Fig. 1. ■, No added substrate; ▲, butyrate (10mM). Concentrations of decanoate: ○, 1mM; △, 2mM; □, 5mM; ⊙, 10mM.

Fig. 1 shows the oxygen consumption by rumen epithelium in relation to the concentration of octanoate. Inhibition of respiration occurred above 2mM, and the loss of activity was progressive. Butyrate (10mM) was also included in the experiment for comparison. At 20mM-octanoate the total oxygen consumed was less than half that observed with only endogenous substrates. Fig. 2 shows a similar experiment in the presence of increasing concentration of decanoate. In this case there was a progressive decline in total oxygen consumption at concentrations above 1mM, and respiration was inhibited to below the endogenous rate at less than 5mM concentration. Table 4 shows that the production of ketone bodies in the presence of increasing concentration of both fatty acids paralleled oxygen consumption. Thus, for the longer chain fatty acids, as shown previously for butyrate (Hird & Weidemann, 1964*b*), the oxygen consumption of the tissue in the presence of the fatty acid is related to the metabolism of fatty acids to ketone bodies. In these experiments the fatty acids were in contact with both surfaces of the tissue. Accordingly it was necessary to study the system in which the normal surface (papillae) was presented to the fatty acid.

Transport experiments. Tables 5 and 6 give the relationship between ketone-body production and the concentration of octanoate and decanoate when these acids were presented to the papillae surface

Table 4. *Effect of fatty acid concentration on oxygen consumption and ketone-body production by isolated rumen epithelium*

Tissue was incubated in Warburg flasks at 38° for 2hr. as described in the text. Results are expressed as μmoles/50mg. dry wt. of tissue/2hr. for duplicate experiments.

	Octanoate		Decanoate	
Concn. of substrate (mM)	Oxygen consumption (μmoles)	Total ketone bodies (μmoles)	Oxygen consumption (μmoles)	Total ketone bodies (μmoles)
0	10·0	0·6	10·5	0·6
	11·3	0·7	10·8	0·6
1	15·5	5·4	14·1	3·4
	18·6	5·9	13·1	2·7
2	16·4	6·8	12·9	3·2
	21·5	8·8	11·1	2·2
5	—	—	6·5	0·4
	—	—	6·3	0·2
10	10·4	2·4	4·0	0·1
	9·7	1·7	5·2	0·01
20	4·0	0·6	—	—
	2·7	0·3	—	—

of rumen epithelium in diaphragm-type experiments. It can be seen that concentrations above 2 mM-octanoate and 1 mM-decanoate gave a progressive decrease in the total ketone bodies appearing on the opposite (muscle) side of the tissue. This effect was more marked with decanoate. When octanoate was applied on the papillae side the inhibition was less than when it was in contact with both surfaces (Table 4). This is consistent with the view that the oxidizing system is closer, or more accessible, to the surface normally attached to the muscle (Hird & Weidemann, 1964*a*). The oxidizing enzymes are therefore likely to be in contact with a higher concentration of the inhi-

Table 5. *Metabolic products formed during the metabolism and transport of octanoate by isolated rumen epithelium*

Conditions were as described for Table 2. Results, calculated as μmoles/diaphragm/2 hr., are given for two separate experiments. See Table 6 for endogenous values. Substrates were added to the papillae surface.

	Gain on muscle side (μmoles)				Gain or loss on papillae side (μmoles)			
		Fatty acid				Fatty acid		
Additions to the papillae side	Ketone bodies	Acetate	Hexanoate	Octanoate	Ketone bodies	Acetate	Hexanoate	Octanoate*
Octanoate (1 mM)	23	2·6	0·1	0·27	4	2·1	0·1	−19
	22	2·9	0·1	0·30	2	0·1	0·1	−17
Octanoate (2 mM)	30	5·6	0·4	3·1	7	2·2	0·8	−28
	32	3·7	0·6	1·2	4	0·2	0·7	−27
Octanoate (5 mM)	21	2·1	0·5	15	6	1·9	1·1	−38
Octanoate (10 mM)	18	3·2	0·5	28	5	2·6	1·1	−53
	20	2·1	0·5	21	2	0·1	0·2	−38
Octanoate (20 mM)	13	2·0	0·4	34	1	0·0	1·0	−63

* The loss of octanoate includes fatty acid metabolized plus the amount remaining in the tissue at the end of the experiment.

Table 6. *Metabolic products formed during the metabolism and transport of decanoate by isolated rumen epithelium*

Conditions and calculations were as described for Table 2. The decanoate added contained 5% of octanoate; it was added to the papillae surface.

	Gain on muscle side (μmoles)				Gain or loss on papillae side (μmoles)			
		Fatty acid				Fatty acid		
Additions to the papillae side	Ketone bodies	Hexanoate	Octanoate	Decanoate	Ketone bodies	Hexanoate	Octanoate	Decanoate*
None	3	0·02	0·00	0·03	0·0	0·03	0·02	−0
	4	0·05	0·04	0·00	0·2	0·04	0·01	−0
Decanoate (1 mM)	14	0·6	0·7	1·9	1	0·5	1·0	−15
	11	0·5	0·7	2·1	1	0·4	1·2	−14
	22	0·4	0·4	1·5	1	0·6	1·1	−18
	20	0·5	0·8	1·9	0·3	0·2	1·8	−16
Decanoate (2 mM)	3	0·3	1·0	8	1	0·4	0·8	−16
	6	0·4	1·2	6	1	0·4	1·7	−17
Decanoate (5 mM)	2	0·3	1·3	19	0·4	0·4	−0·1	−30
	4	0·3	1·2	17	0·1	0·3	−0·1	−26
Decanoate (10 mM)	2	0·0	1·4	28	0·2	0·0	−0·8	−56
	2	0·2	1·6	30	0·2	0·3	−1·7	−43

* The loss of decanoate includes fatty acid metabolized plus the amount remaining in the tissue at the end of the experiment.

bitory fatty acid when this is in contact with the muscle surface.

Analysis of the medium on both sides of the tissue by gas chromatography (Table 5) indicated that small amounts of acetate and hexanoate were also produced during the oxidation of octanoate. These fatty acids of lower molecular weight usually appeared on both sides of the tissue, although more acetate was found on the muscle side than the papillae side. The results suggest the presence of a deacylase(s) acting on intermediates produced during the oxidation of octanoate. The possibility of leakage of acyl-CoA intermediates from the tissue and their subsequent deacylation during the estimation procedure would not account for more than 10% of the lower fatty acids obtained, if amounts of CoA did not exceed those found in sheep liver (Jarrett & Filsell, 1964). Table 5 also indicates that, as the ketone-body production fell off at higher concentrations of the fatty acid, the proportion of unmetabolized fatty acid moving across the tissue increased as the concentration increased.

The results of a similar experiment with decanoate are given in Table 6. In this case also, lower fatty acids were produced by the tissue; the gas chromatograms again showed the formation of acetate, but as this acid was not included in the calibration mixture used for these analyses, it is not possible to give a quantitative figure for its appearance. With decanoate the passage of larger amounts of fatty acid to the muscle side at the higher concentrations coincided with a marked fall in ketone-body production and oxygen consumption. This indicates a simultaneous loss of selective permeability and oxidative processes. The rate of passage of fatty acid across the tissue in the absence of marked ketone-body production (2mM-decanoate and above) appears to be proportional to the concentration of the fatty acid (Table 6).

Production of ketone bodies from long-chain fatty acids

Table 7 shows the effect of adding long-chain fatty acids (1mM) to the papillae side of rumen epithelium. Bovine serum albumin (0·5%) was added to the incubating medium in each case to increase the solubility of the fatty acid. It can be seen that serum albumin alone produced a rise in total ketone-body production above the endogenous level. In the presence of the fatty acids, ketone-body production was consistently higher than that found with serum albumin alone, and the distribution of ketone bodies at the end of the experiment was always in favour of the muscle side. It is possible that the lower activity associated with palmitate and the higher fatty acids was due to decreasing solubility, as solution of fatty acids higher than myristate was incomplete. It is apparent from Table 7 that long-chain fatty acids normally found in the rumen (palmitate, stearate, oleate and linolenate) are, like the shorter-chain fatty acids tested, precursors of ketone bodies during their transport through the tissue.

Table 7. *Ketone-body production accompanying the metabolism of long-chain fatty acids added to the papillae surface of isolated rumen epithelium*

The fatty acids, added to give a concentration of 1mM, were applied to the papillae surface of the epithelium (bearing small papillae). Bovine serum albumin (0·5%) was present in the fatty acid-containing solution. Other conditions are given in the text. Results are the mean of duplicate estimations.

Additions to the papillae side	Appearance of total ketone bodies (μmoles)	
	Muscle side	Papillae side
None	2·2	0·2
	3·8	0·5
Bovine serum albumin (0·5%)	5·7	2·1
	4·7	1·9
	6·1	0·3
	6·2	0·2
	5·4	0·5
	5·2	0·9
Laurate (1 mM)	12·6	0·5
	14·7	0·2
Myristate (1 mM)	14·6	0·2
	17·3	0·6
Palmitate (1 mM)	7·9	0·3
	10·5	1·0
Stearate (1 mM)	6·6	2·7
	7·0	2·1
Oleate (1 mM)	8·1	3·5
	8·9	3·7
Linolenate (1 mM)	8·0	3·8
	6·2	3·5

DISCUSSION

The application of even-numbered fatty acids to the papillae surface of rumen epithelium has been shown, in each case, to lead to the preferential release of ketone bodies on the muscle side of the tissue. Apart from acetate, which is not extensively converted into ketone bodies (Pennington, 1952; Hird & Symons, 1961), the longer-chain fatty acids are transported and metabolized by the tissue in a similar fashion to butyrate (Hird & Weidemann, 1964*a*). In the series acetate to decanoate, there is little transport of the unmetabolized fatty acid unless the concentration of fatty acid in the medium

is raised to a value at which respiration is inhibited and selective permeability is lost. At concentrations of the fatty acids which are not inhibitory, the production of ketone bodies and their selective release on the side of the tissue normally in contact with the blood supply would therefore appear to be a part of the mechanism of absorption of these fatty acids. The slow rate of fatty acid transport (as such) may be related to the pH of the incubating medium (7·4), under which conditions absorption *in vivo* is slow (Danielli, Hitchcock, Marshall & Phillipson, 1946; Gray, 1948).

Experiments with octanoate and decanoate suggest that 'deacylase' activity leads to the production of shorter-chain fatty acids from intermediates formed during oxidation. The existence of deacylase(s) in rumen epithelium is consistent with the observation of Duncombe & Glascock (1954) that labelled volatile fatty acid appears in blood after the feeding of tritiated stearic acid to goats. A similar pathway in the liver of the ruminant is probably partly responsible for the release of radioactive acetate into the blood when labelled palmitate is infused (Connolly, Head & Williams, 1964; West & Annison, 1964).

The known effects of fatty acids on tissue respiration (Hagen & Ball, 1961; Enser, 1964) and oxidative phosphorylation (Björntorp, Ells & Bradford, 1964; Hird & Weidemann, 1966) suggests that the metabolism of these compounds to ketone bodies achieves several useful results. The tissue is able to use the fatty acids as a substrate for its energy requirements (Hird & Weidemann, 1964*b*) and the excess of carbon atoms are available for distribution through the body as ketone bodies etc. In this way deleteriously high concentrations of fatty acids are avoided.

The authors acknowledge financial support from the Commonwealth Bank Rural Credits Development Fund, the Australian Wool Research Committee and the Australian Dairy Produce Research Committee.

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The Effect of 6-Furfurylaminopurine on Senescence in Tobacco-Leaf Tissue after Harvest

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(*Received* 19 *July* 1965)

1. The concentration of chlorophyll decreased and the concentration of α-amino nitrogen and the absolute and specific activity of peptidase increased in disks of tobacco leaf incubated on water in darkness at 25°. 2. Although 6-furfurylaminopurine (kinetin) applied to the disks inhibited the increase in activity of peptidase after 4 days, this effect was too slow to account for the significant inhibition of increase in α-amino nitrogen after 24hr. 3. Treating with kinetin also delayed loss of chlorophyll and soluble protein, though not until 4 and 8 days respectively. 4. The only significant effect of treating disks prepared from tobacco plants deficient in nitrogen with kinetin for 8 days was to inhibit the increase in α-amino nitrogen. 5. Adding kinetin (5mμM to 5μM) *in vitro* did not affect the activity of peptidase extracted from tobacco leaf.

6-Furfurylaminopurine (kinetin) applied to harvested leaves (Richmond & Lang, 1957) or to disks cut from leaves (Osborne, 1962; Sugiura, Umemura & Oota, 1962) delays loss of chlorophyll and protein. As treating with kinetin delays loss of RNA also (Osborne, 1962; Sugiura *et al.* 1962), the primary action of kinetin could be to maintain the mechanism of protein synthesis, a process involving several RNA fractions. However, inhibiting protein degradation by proteolytic enzymes also could be the primary action of kinetin. The experiments described here show that, although treating with kinetin inhibits the increase in specific activity of peptidase occurring in untreated leaf tissue after harvest (Anderson & Rowan, 1965), the effect is too slow to underlie the primary action of kinetin. The first effect observed was inhibition of the increase in concentration of α-amino nitrogen occurring in untreated tissue.

MATERIALS AND METHODS

The preparation of disks of tobacco leaf and the estimation of chlorophyll, α-amino N, soluble protein and activity of peptidase in the tissue were as described by Anderson & Rowan (1965). The chlorophyll in each sample was extracted in 25ml. of ethanol (80%, v/v). Unless stated otherwise, disks were cut from leaves in the early stages of senescence (distinguished from mature leaves by lower chlorophyll content). After harvest, samples of leaf-disks (average mass per disk, 30mg.) were floated abaxial side upwards on 10ml. of glass-distilled water or solutions of kinetin in Petri dishes in darkness at 25°. The water or kinetin solutions were changed daily and did not become cloudy through infection with bacteria. Brown areas appearing on disks treated with water were free of fungal infection when examined under the microscope. The least significant difference between two mean values was calculated by a *t* test after analysis of variance. Kinetin was supplied by L. Light and Co. Ltd., Colnbrook, Bucks.

RESULTS

Effect of kinetin on changes in concentration of α-amino nitrogen and chlorophyll after harvest in disks cut from leaves of increasing physiological age. Three samples of leaf-disks were cut from each of a series of leaves from a mature plant. One sample from each leaf was analysed immediately for α-amino nitrogen and chlorophyll; the other two batches were treated with kinetin (50μM) or water respectively for 7 days. Treating disks cut from leaves of all ages with kinetin delayed degradation of chlorophyll and prevented the increase in α-amino nitrogen found in control disks (Fig. 1). The difference in concentration of chlorophyll in treated and control disks was greatest in disks cut from leaves nos. 10, 11 and 12. Since leaf no. 10 contained half the initial concentration of chlorophyll found in leaves of higher number, light-green leaves were used in subsequent experiments.

Effect of concentration of kinetin and duration of treatment on change in concentration of α-amino nitrogen, chlorophyll and soluble protein, and in activity of peptidase, in leaf tissue after harvest. Treating leaf-disks with kinetin at 1μM and above for 8 days prevented accumulation of α-amino nitrogen, and delayed the degradation of chlorophyll and soluble protein, and the increase in

absolute and specific activity of peptidase (Fig. 2). When samples of disks treated with 50 μM-kinetin were analysed at regular intervals, the concentration of α-amino nitrogen decreased after 2 days but increased in control disks treated with water (Fig. 3); concentrations of 0·1 μM and above inhibited the increase in α-amino nitrogen after 24 hr. (Fig. 4). However, treating with kinetin did not delay degradation of chlorophyll and soluble protein or the increase in activity of peptidase until day 4 or later (Fig. 3), and concentrations of 5 μM and above caused no significant change in these fractions after 3 days.

Effect of nitrogen deficiency on change in concentration of chlorophyll and α-amino nitrogen, and in activity of peptidase, in leaf tissue after harvest. Plants were grown in soil and transferred to a complete liquid culture medium after 10 weeks. After a further 7 weeks, a matched pair of plants was transferred to a medium containing no nitrogen and a second matched pair was kept as a control in complete medium. Leaf-disks were cut from deficient and control plants 20 days after the transfer of the first pair of plants to deficient medium. Five samples per plant containing 20 disks each were prepared from leaves appearing to contain equal amounts of chlorophyll per unit area. One sample from each plant was analysed immediately and the remaining samples were analysed after treating either with water or with kinetin (50 μM) for 4 and 8 days. In disks from plants cultured in nitrogen-deficient medium for 20 days, treating with kinetin did not significantly delay loss of chlorophyll or the increase in activity of peptidase found in control disks from nitrogen-sufficient plants (Fig. 5). However, treating with kinetin prevented increase in α-amino nitrogen in both sufficient and deficient disks after 4 days.

Changes in α-amino nitrogen in incubated disks examined by paper chromatography. The concentration of α-amino nitrogen in samples of leaf-disks on the day of harvest increased after incubating on water for 2 days, but decreased after incubating with 50 μM-kinetin. Samples (10 μl.) of the extracts in which α-amino nitrogen was determined were applied to Whatman no. 1 paper with appropriate marker spots of amino acids, and developed in butan-1-ol–acetic acid–water (4:1:1, by vol.).

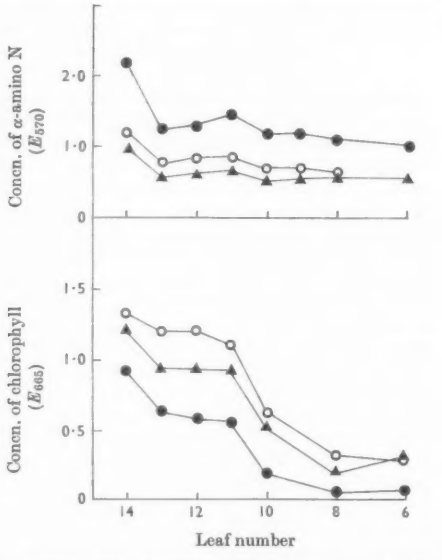

Fig. 1. Effect of kinetin (50 μM) on changes in concentration of α-amino N and chlorophyll after harvest in disks cut from leaves of increasing physiological age. After incubating for 7 days, the samples containing 16 disks were ground in 3·0 ml. of extracting medium. Day 0: ○. Day 7: incubated on water, ●; incubated on kinetin, ▲.

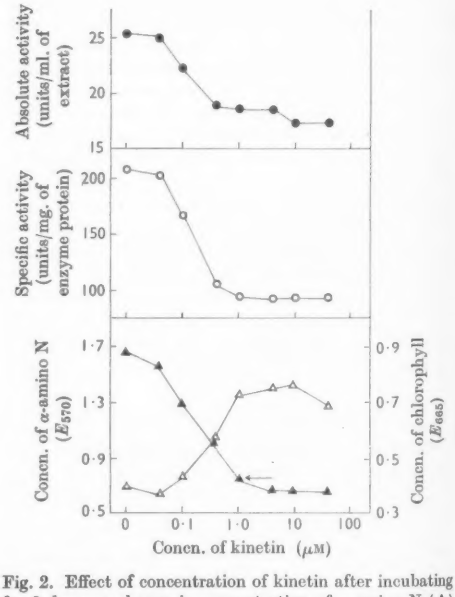

Fig. 2. Effect of concentration of kinetin after incubating for 8 days on change in concentration of α-amino N (▲) and chlorophyll (△), and in absolute (●) and specific activity (○) of peptidase. The arrow shows the concentration of α-amino N at harvest. Samples containing 26 disks were ground in 3·0 ml. of extracting medium. All results are means of duplicate extractions.

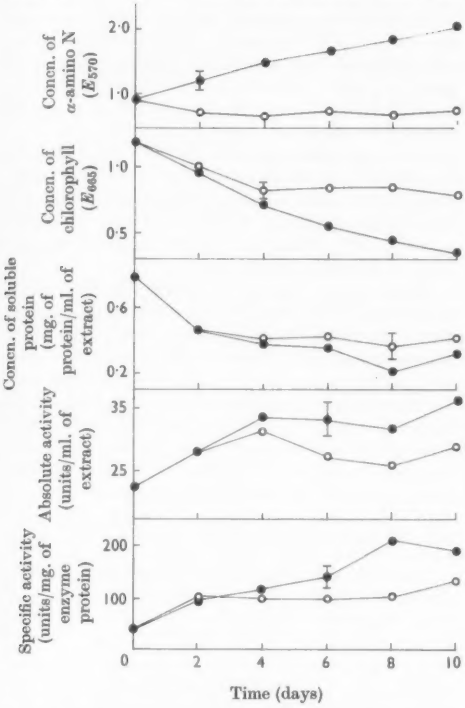

Fig. 3. Effect of kinetin on changes in concentration of α-amino N, chlorophyll and soluble protein and in absolute and specific activity of peptidase in leaf-disks with time after harvest. Samples of 25 disks were incubated on water (●) or 50 μM-kinetin (○) and ground in 3·0 ml. of extracting medium. Vertical lines show the least significant difference (P 0·05) between two mean values. All results are means of duplicate extractions.

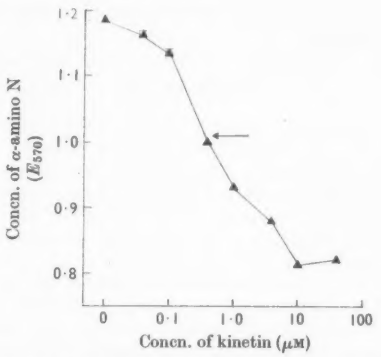

Fig. 4. Effect of concentration of kinetin after incubating for 24 hr. on change in concentration of α-amino N. The arrow shows the concentration of α-amino N at harvest. Samples containing 10 disks were ground in 2·1 ml. of extracting medium. Results are means of duplicate extractions.

Replicate chromatograms run for 12 and 53 hr. were sprayed with ninhydrin or with the peptide spray of Rydon & Smith (1952). The predominant spots were glutamic acid, and peptides remaining at or close to the start line. Material with R_F equal to glutamic acid was eluted with water and the identification confirmed by chromatography in propan-2-ol–water (7:3, v/v). The intensity of all spots increased on chromatograms of extracts prepared after treating with water (Ranjan & Laloraya, 1960), but decreased after incubating on kinetin.

Effect of kinetin on the activity of peptidase in vitro. The activity of peptidase was measured in reaction mixtures containing kinetin between 5 mμM and 5 μM. Including kinetin in the reaction

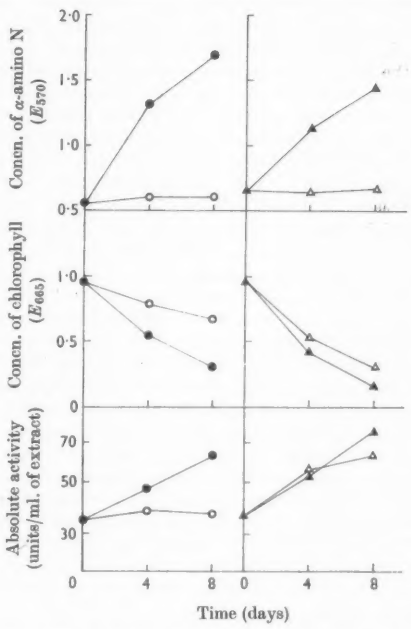

Fig. 5. Effect of kinetin on changes in concentration of α-amino N, chlorophyll and activity of peptidase in leaf-disks cut from tobacco plants grown with sufficient and deficient N. Samples containing 20 disks were incubated on water (with N, ●; without N, ▲) or 50 μM-kinetin (with N, ○; without N, △), and ground in 3·0 ml. of extracting medium. Results are means from duplicate plants.

mixtures did not change the activity of peptidase significantly.

DISCUSSION

In the experiments described here the most rapid response to treating leaf-disks of tobacco with kinetin was found in α-amino nitrogen. The effect on this fraction varied with the concentration of kinetin and the length of the treatment. In short experiments (24hr.), 0·5 μM-kinetin prevented the increase in α-amino nitrogen found in the controls (Fig. 4), but concentrations of 1·0 μM and above caused a decrease in α-amino nitrogen. The effect was essentially the same in longer experiments (8 days), but the decrease in α-amino nitrogen at high concentrations of kinetin (5·0 μM or more) was small compared with the increase in the absence of kinetin (Fig. 2). In both long and short experiments, concentrations of kinetin above 10 μM caused no further decrease in α-amino nitrogen.

Treating with kinetin did not delay significantly the increase in activity of peptidase and the decrease in concentration of chlorophyll and soluble protein until 4 days after harvest (Fig. 3). In disks from nitrogen-deficient plants, inhibition of the increase in α-amino nitrogen was the only significant effect of treating with kinetin for 8 days (Fig. 5). Since treating with kinetin inhibits the increase in α-amino nitrogen without preventing increase in peptidase activity, we conclude that kinetin delays protein degradation in harvested leaf tissue by stimulating protein synthesis rather than inhibiting degradation of protein by peptidase. Thus the decrease in α-amino nitrogen in disks treated with high concentrations of kinetin (Figs. 1–4) represents utilization in protein synthesis.

Since treating with kinetin inhibited the increase in α-amino nitrogen in harvested tissue, though it did not prevent soluble protein decreasing (Fig. 3), we conclude that the treatment induces synthesis of protein insoluble at pH 5·2. Decrease in glutamic acid and peptides observed on chromatograms of extracts prepared from tissue treated with kinetin suggests that these are major substrates for synthesis of this protein. Although protein of tobacco leaf contains 13–20% (w/w) of glutamic acid (Tso & McMurtrey, 1960), we cannot be certain if this amino acid is incorporated into protein directly, or donates α-amino nitrogen for synthesis of other amino acids.

The delay in increase of α-amino nitrogen caused by treating with kinetin for 24hr. increases gradually between 0·05 and 10 μM (Fig. 4), and could provide a more rapid bioassay for kinins than the delay in degradation of chlorophyll proposed by Osborne & McCalla (1961).

Although adding kinetin *in vitro* has activated such hydrolytic enzymes as ribonuclease and deoxyribonuclease (Maciejewska-Potapczyk, 1959) and nucleotidase (Shuster & Gifford, 1962), this treatment did not affect the activity of the peptidase extracted from tobacco leaf.

Mothes and co-workers (Mothes, Engelbrecht & Kulajewa, 1959; Mothes, Engelbrecht & Schütte, 1961; Parthier, 1961) believed that amino acids accumulating in the area of a harvested leaf treated with kinetin (kinetin-directed transport) increased the rate of synthesis of protein in the area. In the experiments reported here, treating with kinetin delayed senescence in leaf-disks where kinetin-directed transport cannot take place. If the amount of amino acids in harvested leaves regulated the rate of synthesis of protein, decrease in concentration of protein would never occur.

This work was supported by a grant from the Rural Credits Development Fund of Australia.

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Purification of β-Acetylglucosaminase and β-Galactosidase from Ram Testis

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(*Received* 2 *July* 1965)

1. The presence of β-galactosidase (EC 3.2.1.23) in an acetic acid extract of ram testis is reported. Some properties of the crude enzyme preparation were studied. 2. The purification of β-acetylglucosaminase (EC 3.2.1.30) and of β-galactosidase from the ram-testis extract by ammonium sulphate precipitation and chromatography on a CM-cellulose column is described. 3. The final purifications of the separated enzymes achieved were for the β-acetylglucosaminase 35 times and for the β-galactosidase 99 times. 4. The possibility of using DEAE-cellulose and Sephadex G-200 to purify the enzymes was investigated.

Although the protein portion of ovomucoid has been degraded by proteolytic enzymes [pancreatic enzymes and trypsin (Tanaka, 1961*a*,*b*,*c*,*d*), pepsin (Hartley & Jevons, 1962), Pronase (Marks, Marshall, Neuberger & Papkoff, 1962) and papain (Montgomery & Wu, 1963)], little work on the enzymic degradation of the carbohydrate moiety has been reported. In a preliminary communication Caygill & Jevons (1964) reported the slow release of *N*-acetylglucosamine from the carbohydrate moiety of ovomucoid by a crude extract of ram testis. As the ram-testis extract was known to contain a β-acetylglucosaminase (Borooah, Leaback & Walker, 1961), further purification of this enzyme was considered desirable to investigate whether it was responsible for the release of *N*-acetylglucosamine from ovomucoid, and whether a purer preparation would change the quantity and rate of release. During the course of this purification, β-galactosidase, for which ovomucoid could be a substrate, was detected.

MATERIALS AND METHODS

p-Nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside was synthesized by the method of Leaback (1963) (m.p. 216–218°, $[\alpha]_D^{30}$ −17°, *c* 0·5% in water), or was bought from Koch–Light Laboratories Ltd., Colnbrook, Bucks. *o*-Nitrophenyl β-D-galactopyranoside was obtained from Koch–Light Laboratories Ltd., and *p*-nitrophenyl β-D-galactopyranoside from British Drug Houses Ltd., Poole, Dorset.

CM-cellulose, prepared by the method of Peterson & Sober (1956), was kindly supplied by Dr C. H. Wynn, and DEAE-cellulose was purchased from Eastman–Kodak Ltd., Kirkby, Liverpool. Sephadex G-200 (in the bead form) was obtained from Pharmacia, Uppsala, Sweden.

Bovine plasma albumin was obtained from Armour Pharmaceuticals Ltd., Eastbourne, Sussex, and *o*-nitrophenol was synthesized in this Department.

An extract of ram testis, prepared by mincing 500g. of the unskinned organs, and extracting with 2l. of acetic acid at 0–5°, was a gift from Fisons Pharmaceuticals Ltd., Holmes Chapel, Cheshire.

Estimation of enzymic activities. β-Acetamido-2-deoxy-D-glucoside acetamidoglucohydrolase (EC 3.2.1.30) (β-acetylglucosaminase) activity was estimated by the method of Woollen, Heyworth & Walker (1961) with *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (final concn. 3·6mM). Bovine plasma albumin (0·2%) was included in all assays, as recommended by the above-named authors.

β-Galactoside galactohydrolase (EC 3.2.1.23) (β-galactosidase) was estimated by a slight modification of the method of Lederberg (1950), with the synthetic substrates *o*- and *p*-nitrophenyl β-D-galactopyranoside. Duplicate mixtures containing, except where otherwise indicated, *o*-nitrophenyl galactoside (final concn. 4·0mM) or *p*-nitrophenyl galactoside (final concn. 2·0mM), and enzyme solution diluted as appropriate, in 0·05M-citric acid–sodium citrate buffer, pH4·5, to give final vol. 1·0ml., were incubated at 37°. The reaction was stopped by the addition of 0·2M-sodium tetraborate adjusted to pH9·8 with 0·2N-NaOH (2·0ml.), to give final pH9·2. The extinction at 400mμ was measured in a Unicam SP.500 spectrophotometer, and the quantity of aglycone liberated determined from the appropriate calibration curve. In those experiments where the assay was carried out in buffers of different pH values the final pH was adjusted to pH9·2 (where necessary) by the addition of 1 to 3 drops of N-NaOH or N-citric acid or N-acetic acid.

The details of the purification of β-acetylglucosaminase

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and β-galactosidase by precipitation with $(NH_4)_2SO_4$ and column chromatography on CM-cellulose are given with the relevant results.

RESULTS

As no report of the properties of ram-testis β-galactosidase has been found, some properties were studied to ascertain the best conditions in which to assay the enzyme. Linear reaction rates were recorded over a 10-fold range of enzyme concentrations, with times of incubation up to 90min.

Effect of pH and substrate concentration on β-galactosidase activity. Fig. 1 shows the effect of pH on β-galactosidase activity with *o*-nitrophenyl β-D-galactoside as substrate. Similar results were obtained with 2mM-*p*-nitrophenyl β-D-galactoside, 0·1ml. of a fourfold-diluted extract, incubated for 15min. in 0·05M-citrate or 0·05M-acetate buffer, being used.

Fig. 2 shows the variation of β-galactosidase activity with concentration of *o*-nitrophenyl β-D-galactoside. It will be noted that the enzyme was inhibited by excess of substrate. The double-reciprocal plot attributed to Lineweaver & Burk (1934) gives the apparent K_m 0·67mM. A similar shaped curve, showing slight inhibition by excess of substrate, was obtained in 0·05M-acetic acid–sodium acetate buffer, pH4·5, when *p*-nitrophenyl β-D-galactoside was used as substrate.

The apparent K_m with *o*-nitrophenyl β-D-galactoside as substrate was also 0·67mM when measured in 0·1M-citric acid–sodium phosphate buffers of pH6·95, 6·7, 6·3, 5·9, 5·0 and 4·05, or in 0·05M-acetic acid–sodium acetate buffers of pH6·7, 5·5, 5·05, 4·5, 4·3 and 3·5. At pH2·7, in the former buffer, K_m 0·15mM was obtained. A value for the apparent K_m 0·15mM was found with *p*-nitrophenyl β-D-galactoside as substrate in 0·1M-citric acid–sodium phosphate buffers of pH5·5, 4·45 and 3·4.

Purification of β-acetylglucosaminase and β-galactosidase. (*a*) Ammonium sulphate fractionation. Ram-testis extract was fractionated by the addition of solid ammonium sulphate, and the results are shown in Table 1. The fraction precipitating between 30 and 40% saturation with ammonium sulphate (fraction P_{40}), which had specific activities of 7·1 times (β-acetylglucosaminase) and 6·2 times (β-galactosidase) that of the ram-testis extract, was used for the next stage of the purification.

(*b*) Chromatography on a CM-cellulose column. CM-cellulose (5g.) was added to 150ml. of 0·05N-sodium hydroxide–0·05M-sodium chloride (1:1, v/v). This was poured into a Buchner funnel, and washed without suction with 2l. of this mixture followed by 2l. of 0·1N-hydrochloric acid and 0·05M-citrate buffer, pH4·5, until no Cl^- ions were detected in the washings. A slurry of the CM-cellulose in the citrate buffer was poured into a column (40cm. by 1·5cm. diam.). Dialysed fraction

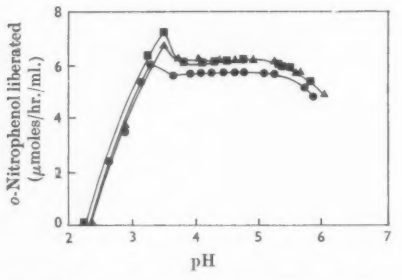

Fig. 1. pH–activity curve of β-galactosidase. Incubation mixtures (1·0ml.), incubated for 30min. at 37°, contained 0·25ml. of diluted ram-testis extract (1:2), 4·0 mM-*o*-nitrophenyl β-D-galactopyranoside in 0·05M-buffers of various pH values: ■, citric acid–sodium citrate; ●, acetic acid–sodium acetate; ▲, citric acid–disodium hydrogen phosphate.

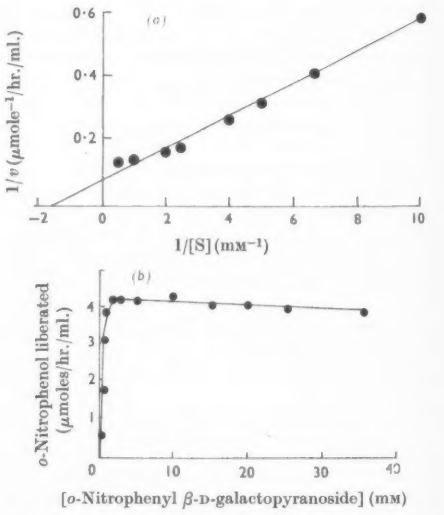

Fig. 2. Effect on β-galactosidase activity of varying substrate concentration. The β-galactosidase activity of 0·25ml. of diluted ram-testis extract (1:2) was determined by incubation in 0·05M-citric acid–sodium citrate buffer, pH4·5, for 30min., with *o*-nitrophenyl β-D-galactopyranoside of the concentrations shown: (*a*) as a double reciprocal plot of 1/[substrate] against 1/activity; (*b*) as a plot of activity against substrate concentration.

Table 1. *Ammonium sulphate fractionation of ram-testis extract*

Solid $(NH_4)_2SO_4$ was added at 4° to the ram-testis extract, and stirred for 40 min. Precipitates were redissolved in water and dialysed against four changes (20 vol.) of distilled water, followed by two changes (10 vol.) of 0·05 M-citrate buffer, pH 4·5, for 12 hr. in each case. Enzyme activities were estimated as described in the text. Protein was estimated by measuring the extinction at 280 mμ. Enzyme activity/ml. is expressed as μmoles of aglycone liberated/hr./ml. and specific activity as activity/ml./E_{280} unit.

	β-Acetylglucosaminase			β-Galactosidase			
Fraction	Activity/ml.	Specific activity	Recovery of enzyme activity (%)	Activity/ml.	Specific activity	Recovery of enzyme activity (%)	Recovery of protein (%)
Ram-testis extract	216	14·1	100	7·26	0·47	100	100
0–20% satn. $(NH_4)_2SO_4$ ppt.	70	7·8	3	3·77	0·42	5	6
20–30% satn. $(NH_4)_2SO_4$ ppt.	107	21·2	5	3·59	0·69	5	4
30–40% satn. $(NH_4)_2SO_4$ ppt.	1761	100·0	81	48·40	2·75	63	17
40–45% satn. $(NH_4)_2SO_4$ ppt.	57	8·4	3	0·92	0·13	1	11

Table 2. *Chromatography on carboxymethyl-cellulose*

Recovery, expressed as % of material applied to the column, of β-acetylglucosaminase and β-galactosidase activities, and of protein, from a CM-cellulose column eluted as described in Fig. 3.

	Recovery (%)		
Fraction (tube nos.)	β-Acetyl-glucosaminase	β-Galacto-sidase	Protein
5– 8	0	65·5	8·4
35–37	56·2	5·5	13·8
63–65	30·6	0	24·8
Total	94·5	79·0	90·0

P_{40} (5·0 ml.) was washed into the column with 0·05 M-citrate buffer, pH 4·5. The column was then eluted with a series of buffers of increasing pH as indicated in Fig. 3. Table 2 shows the recovery of the two enzymes and protein in the tubes containing most enzyme, as a percentage of that applied to the column. The highest purification factors obtained were for β-galactoside, 15·9 times (tube 6), and for β-acetylglucosaminase, 4·9 times (tube 36).

Fig. 3 also shows that the β-acetylglucosaminase activity is eluted in two peaks, and the intervening tubes contain little enzyme activity. Rechromatography of the second peak (which had been eluted by buffer pH 5·5) showed that no activity was eluted by buffer pH 4·9, the pH at which the major portion (67%) of the activity was eluted from the first column, but was again eluted by buffer pH 5·5. It would thus appear that the β-acetylglucosaminase activity present can be separated into two fractions, which are eluted from CM-cellulose by buffers of different pH.

(c) Investigation of alternative purification procedures. β-Acetylglucosaminase was adsorbed by DEAE-cellulose from 0·02 M-sodium dihydrogen phosphate–disodium hydrogen phosphate buffer, pH 7·5, and could be eluted by 0·05 M-citrate buffer, pH 3·0, containing a final concentration of 0·67 M-sodium chloride. When an attempt was made to elute β-acetylglucosaminase from a DEAE-cellulose column, no activity was recovered, despite the recovery of 102% of the added protein, as estimated by the extinction at 280 mμ. However, the enzyme would be expected to be inactivated at pH 3·0, in the time taken to elute it from a column. Fraction P_{40} (0·2 ml.) was incubated at 20° in 0·05 M-citrate buffer (1·8 ml.) of various pH values from 2·0 to 9·0, and 0·1 ml. samples were withdrawn at intervals, diluted with 0·9 ml. of 0·05 M-citrate buffer, pH 4·5, and 0·1 ml. of this was used to estimate residual β-acetylglucosaminase activity. Between pH 4·0 and pH 9·0 the β-acetylglucosaminase was relatively stable for up to 2 hr., but activity was rapidly lost in more acid solutions. Findlay & Levvy (1960) reported that pig-epididymal β-acetylglucosaminase was stable in the range pH 4·0–8·0 for 1 hr. at 37°, but lost all its activity in this time at pH 2·2.

β-Acetylglucosaminase in 0·05 M-citrate buffer, pH 4·5, was absorbed at room temperature by sand previously washed with 2 N-hydrochloric acid followed by 0·1 N-sodium hydroxide and distilled water, and 75% of the absorbed enzyme could be eluted with 0·02 M-phosphate buffer, pH 7·5.

The use of Sephadex G-200 was investigated. Sephadex G-200 (5 g.) was equilibrated for at least 4 days with 0·05 M-citrate buffer, pH 4·5, and poured into a column (40 cm. by 3 cm. diam.). Fraction P_{40} (5·0 ml.) was applied to this column, and then eluted with 0·05 M-citrate buffer, pH 4·5. The acetylglucosaminase activity appeared in the

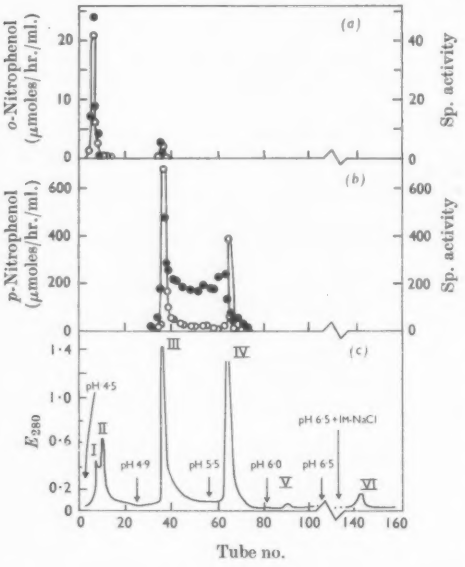

Fig. 3. Chromatography of fraction P_{40} on CM-cellulose. Fraction P_{40} was applied to a CM-cellulose column and eluted with a series of 0·05 M-citric acid–sodium citrate buffers, of increasing pH as indicated. The initial pH was 4·5 and the pH was changed as shown by arrows. Fractions (5 ml.) were collected. (*a*) Activity/ml. (○) and specific activity (●) of β-galactosidase. (*b*) Activity/ml. (○), and specific activity (●) of β-acetylglucosaminase. (*c*) Protein, estimated by extinction at 280 mμ, plotted against tube no. (5·0 ml. fractions). No protein was detected in tubes 100–130.

first protein fraction (75–100 ml. of eluate), but the extinction at 280 mμ indicated that some protein was retarded. The recovery of β-acetylglucosaminase from the column was 79%, and of protein 99%, and the tube having the highest specific activity showed a purification factor of only 2·4. However, when a mixture from the tubes from the CM-cellulose column containing the β-acetylglucosaminase with the highest specific activity (tubes 36 and 37, 2·5 ml. of each, adjusted to pH 4·5 with 0·05 M-citric acid) was applied to an identical column of Sephadex G-200, only a small amount of protein devoid of β-acetylglucosaminase activity was eluted with 75–100 ml. of eluent. The major protein-containing fraction was eluted with 140–200 ml. of buffer, and this fraction included the only β-acetylglucosaminase activity eluted from the column. This fraction accounted for 105% of the applied protein, but only 25% of the applied enzyme activity, and had a lower specific activity than the applied material.

DISCUSSION

β-Galactosidase activity with *o*- or *p*-nitrophenyl galactoside as substrate, when plotted against pH, showed a sharp peak at approximately pH 3·5, followed by a plateau of uniform activity from pH 4·0 to 5·2. A double peak, showing maximum activity at approximately pH 2·9 and pH 3·5, was obtained by Conchie & Hay (1959) with phenyl β-D-galactoside in the assay of rat-epididymal β-galactosidase. With *o*-nitrophenyl β-D-galactoside they obtained single peaks at pH 2·9 or at pH 3·5 in acetate or citrate–phosphate buffers respectively. Levvy & McAllan (1963) found maximum activity at pH 2·9 for the rat-epididymal enzyme with *p*-nitrophenyl β-D-galactoside in citrate–phosphate buffer. For the experiments reported here pH 4·5 was selected, as at this pH the enzyme in the crude extract showed no variation in activity with minor variations of pH.

Inhibition by concentrations of substrate above 2 mM (*o*-nitrophenyl β-D-galactoside) and 4 mM (*p*-nitrophenyl β-D-galactoside) is reported. Slight inhibition of rat-epididymal β-galactosidase by excess of substrate was found at concentrations above 5 mM (*o*-nitrophenyl β-D-galactoside) or 15 mM (*p*-nitrophenyl β-D-galactoside) by Conchie & Hay (1959). The values for the apparent Michaelis–Menten constant, K_m, obtained were 0·67 mM with *o*-nitrophenyl β-D-galactoside and 0·15 mM with *p*-nitrophenyl β-D-galactoside, compared with values for the rat-epididymal enzyme of 0·38 mM and 0·27 mM respectively (Levvy & McAllan, 1963).

Ammonium sulphate fractionation appears to be a suitable first step in the purification from ram-testis extract of β-acetylglucosaminase (81% recovery) and β-galactosidase (63% recovery). Chromatography on CM-cellulose then gave good separations, so that the two stages of purification gave enzymes having specific activities 35 times (β-acetylglucosaminase) and 99 times (β-galactosidase) that of the original ram-testis extract.

The β-acetylglucosaminase activity was absorbed at pH 4·5; most was eluted at pH 4·9, and a little at pH 5·5. As on rechromatography of this second peak the β-acetylglucosaminase activity was eluted, not at pH 4·9, but again at pH 5·5, it is possible that the enzymic activity is associated with two different proteins, or one protein existing in two forms which behave differently on CM-cellulose.

Two peaks (at pH 4·5 and pH 4·9 respectively) containing β-galactosidase activity were eluted. As no activity is present in the intervening tubes, in spite of an intervening protein peak, it is possible that these activities are also caused by two different

proteins. If this is so the pH optimum curve obtained with the ram-testis extract could be the result of two separate curves. On the other hand, the finding of the same K_m between pH 6·9 and pH 3·5 for *o*-nitrophenyl β-D-galactoside favours the alternative hypothesis that the activity is due to two forms of the same protein, which behave differently on CM-cellulose. It is unlikely that the same protein in peak III (pH 4·9) is responsible for both the β-galactosidase and β-acetylglucosaminase activities, as the ratio of the two enzymic activities in tubes 36, 37 and 38 (Fig. 3) is not constant.

We are indebted to the Medical Research Council for a grant, and to Miss K. Broady for technical assistance with some of the experiments.

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The Chemical Synthesis and Biological Oxidation of 7α-Hydroxy[26-^{14}C]cholesterol, 7-Dehydro[26-^{14}C]cholesterol and 26-Hydroxy[26-^{14}C]cholesterol

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(*Received* 2 *August* 1965)

1. 26-Hydroxycholesterol was obtained by reducing the methyl ester of (±)-3β-hydroxycholest-5-en-26-oic acid, which was synthesized from 25-oxonorcholesterol. 2. Methods for preparing 7α-hydroxycholesterol and 7-dehydrocholesterol were modified to allow the micro-scale preparation of these [^{14}C]sterols from [26-^{14}C]-cholesterol. 3. 26-Hydroxycholesterol was oxidized more readily than 7α-hydroxy-cholesterol, 7-dehydrocholesterol or cholesterol by mitochondrial preparations from livers of mice, rats, guinea pigs, common toads (*Bufo vulgaris*) and *Caiman crocodylus*. 4. (±)-3β-Hydroxy[26-^{14}C]cholest-5-en-26-oic acid was oxidized very rapidly to $^{14}CO_2$ by mouse and guinea-pig mitochondria without evident discrimination between the two optical isomers. 5. An enzyme system that oxidizes 26-hydroxycholesterol to 3β-hydroxycholest-5-en-26-oic acid was identified in the soluble extract of rat-liver mitochondria. This enzyme could use NADP in place of NAD but was not identical with liver alcohol dehydrogenase (EC 1.1.1.1). 6. [26-^{14}C]Cholesteryl 3β-sulphate was not oxidized by fortified mouse-liver preparations that oxidized [26-^{14}C]cholesterol to $^{14}CO_2$.

Cholesterol is oxidized in the liver of mammals, yielding bile salts, i.e. glycine- or taurine-conjugated hydroxycholanic acids. There is evidence that this overall catabolic process is subject to end-product inhibition (Beher & Baker, 1959; Beher, Anthony & Baker, 1959; Bergström & Danielsson, 1958). Bergström (1959) suggested that bile salts present in the enterohepatic circulation may inhibit the 7α-hydroxylation of cholesterol, believed to be one of the first points of attack on the cholesterol molecule. 7α-Hydroxycholesterol (cholest-5-ene-3β,7α-diol) is formed enzymically from cholesterol on incubation with liver microsomes (Danielsson, 1964). On the other hand, 26-hydroxycholesterol (cholest-5-ene-3β,26-diol) has been isolated from incubations of [^{14}C]cholesterol with liver homogenates and liver mitochondria (Fredrickson & Ono, 1956; Danielsson, 1961) and was the principal hydroxylation product formed from cholesterol in these experiments.

We have attempted to resolve this question as to which hydroxylation reaction might be the early pacemaker in cholesterol catabolism and possibly subject to negative-feedback control. We compared the rates of oxidation of 7α-hydroxycholesterol and 26-hydroxycholesterol, with that of cholesterol itself, by an enzyme system ('cholesterol oxidase'), present in mammalian liver mitochondria, which cleaves the terminal isopropyl group of the sterol side chain and oxidizes the methyl groups to carbon dioxide (Whitehouse, Staple & Gurin, 1959). For this purpose it was necessary to prepare each of these hydroxycholesterols labelled with ^{14}C in the terminal carbon atom (C-26).

A new route for synthesizing (±)-26-hydroxycholesterol has been developed and is described here in some detail.

EXPERIMENTAL

All radioactive compounds and reactions were handled in the absence of direct light and under N_2 where possible. [^{14}C]Sterols were stored in frozen benzene solution at −5° in the dark. Radioactivity was measured by using a Tritomat (Isotope Developments Limited, Beenham, Reading) liquid-scintillation counter after solution in Diotol (Herberg, 1960) containing 2% (w/v) of Cab-o-sil (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.). Counting conditions were arranged to give a standard error for the mean count of a sample of less than 0·7%.

The radiochemical purity of all sterol substrates was checked by radioautography. Thin-layer chromatograms of radioactive sterols and non-radioactive reference compounds were pressed on to Kodak X-ray film and left for 24hr. The non-radioactive markers were then made visible as described below: in all cases only one radioactive spot was detected and corresponded exactly to the appro-

priate non-radioactive marker, or was exactly superimposable with the admixed non-radioactive compound.

Biochemical

Incubation of [26-^{14}C]sterols with liver preparations. Mitochondrial fractions isolated from homogenates of liver from female guinea pigs and female mice were incubated with [26-^{14}C]sterols, emulsified with Tween 20 (Atlas Powder Co., Wilmington, Del., U.S.A.), and the following cofactors: ATP, NAD, sodium citrate, $MgCl_2$, AMP, GSH and a boiled supernatant fraction ('SF'), exactly as described by Lee & Whitehouse (1963). Light was excluded during the period of incubation.

The $^{14}CO_2$ evolved was trapped in centre wells containing 0·2ml. of 2N-KOH or 25% (v/v) triethanolamine in water.

Microsomes were collected by centrifuging at 100000*g* for 30min. the supernatant layer obtained on removing the mitochondrial fraction of liver homogenates (by centrifugation at 8500*g* for 10min.).

Extraction of 26-hydroxycholesterol dehydrogenase from mitochondria. Mitochondrial fractions isolated from liver from female rats as described by Lee & Whitehouse (1963) were dispersed in 0·25M-tris hydrochloride buffer solution, pH8·5 (at 0°), containing 10% (w/v) of sucrose, kept for 5min. at 0° and then centrifuged at 8500*g* for 10min. to remove the particulate fraction, which was then twice washed with the same hyperosmotic buffer–sucrose solution. The soluble extract, the washed mitochondrial particulate fraction and other liver fractions were each incubated with 26-hydroxy[26-^{14}C]cholesterol, emulsified with Tween 20 in 0·25M-tris hydrochloride buffer, pH8·1 (at 37°), with NAD or NADP (1·5mg./incubation mixture) in a final volume of 2ml. for 1hr. with shaking in air at 37°.

Other preparations. Rat-spleen and rat-kidney homogenates were prepared as for liver; heart-muscle homogenates were prepared from chopped rat hearts by prolonged (1min.) homogenizing in a chilled stainless-steel homogenizer.

Mitochondrial fractions and boiled SF were prepared from toad and cayman livers by exactly the same procedure used to prepare mouse-liver 'mitochondria' and SF. Toads (*Bufo vulgaris*, weighing approx. 15g.) were collected from the Norfolk Broads and fed on maggots until used; South American caymans (*Caiman crocodylus*), approx. 36cm. long, were purchased from the Goldfish Bowl, Eastern Avenue, Oxford, and fed on uncooked cattle liver, live minnows or cooked meat purees (baby foods) until used.

Protein determinations were carried out by using a biuret reagent (Gornall, Bardawill & David, 1949).

Extraction and assay of steroid acids. [^{14}C]Steroid acids were estimated by thin-layer chromatography, as follows. Incubations were terminated by the addition of 12N-H_2SO_4 (0·2ml.) and extracted first with ethyl acetate (2×1ml.) and then, after the addition of methanol (1ml.), extracted again with chloroform (1ml.). The organic extracts were combined, washed once with water (1ml.) and concentrated by warming (to 45°) under N_2 to 50μl. This concentrate was applied as a single spot on a thin-layer plate of either alumina (to assay the relative amounts of sterol and steroid acids) or silica gel H (to characterize the steroid acids). The alumina plates were developed to a height of 10cm. with ethyl acetate–light petroleum (b.p. 60–80°) (1:1, v/v). The silica plates were developed with system no. 1 (Hofmann, 1962). Areas corresponding to known steroids were transferred to glass vials and Diotol (4ml.) was added for liquid-scintillation counting.

Other biochemical studies. Crystalline horse-liver alcohol dehydrogenase (alcohol–NAD oxidoreductase, EC 1.1.1.1) (Dalziel, 1961), at 0·55μg./ml., was incubated with 200μg. of sterol/ml., added in 0·1ml. of dimethylformamide–0·5mM-NAD–44mM (0·1*M*)-phosphate buffer, pH8·0, and the enzyme activity assayed fluorimetrically (Dalziel, 1962).

Chemical

All melting points were determined on a Kofler block and are uncorrected.

Thin-layer chromatographic analyses were carried out by using 8 in.×2 in. glass plates coated with silica gel grade H (nach Stahl). Then 5–10μl. of a 1% solution of the compounds to be analysed was adsorbed on the plates and developed with one of the following solvent systems: *A*, ethyl acetate alone; *B*, ethyl acetate–light petroleum (b.p. 60–80°) (1:1, v/v); *C*, ethyl acetate–light petroleum (b.p. 60–80°) (3:7, v/v); *D*, ethanol-free chloroform–ethyl methyl ketone (4:1, v/v). Compounds were detected on the plates by spraying with 10% (w/v) phosphomolybdic acid solution in ethanol or by spraying with 5% (w/v) $SbCl_3$ solution in chloroform, and heating at 110° for 5min. Ketones were detected by spraying with 5% (w/v) 2,4-dinitrophenylhydrazine in methanol–conc. H_2SO_4 (8:1, v/v) (Brady's reagent).

Infrared spectra and optical rotations were determined in chloroform solution, unless otherwise stated. The spectra were calibrated with a polystyrene strip.

Elemental analyses were carried out by Bernhardt and Co., Mulheim, Germany, or by Weiler and Strauss, Oxford.

25-Oxonorcholesterol (3β-hydroxy-27-norcholest-5-en-25-one) (I) was supplied by Schering Corp., Bloomfield, N.J., U.S.A. Sodium [^{14}C]cyanide (specific activity 16·3mc/m-mole), sodium [2-^{14}C]propionate (specific activity 4·2mc/m-mole) and [^{14}C]methyl iodide (specific activity 29·5mc/m-mole) were supplied by The Radiochemical Centre, Amersham, Bucks.

The nuclear-magnetic-resonance (n.m.r.) spectrum was determined by Mrs R. E. Richards, of the Dyson Perrins Laboratory, Oxford.

Dried pyridine. AnalaR pyridine was heated under reflux over KOH for 8hr., then distilled from fresh KOH, with suitable precautions to protect the distillate from atmospheric moisture.

Thionyl bromide. Thionyl bromide, b.p. 38–40°/15mm. Hg, was prepared by the method of Frazer & Gerrard (1954).

Preparation of 3β-hydroxycholest-5-en-26-oic acid (VIII) (*Scheme* 1). A solution of 25-oxonorcholesterol (I) (25g.) in distilled pyridine (240ml.) was treated with acetic anhydride (60ml.). The solution was stirred and heated under reflux for 1hr. The reaction mixture was allowed to cool and poured with stirring into ice and water (about 2l.). The white precipitate that formed was filtered off at the pump and washed successively with dil. HCl and then with water, until free from pyridine. The precipitate was dried and recrystallized from ethanol–water; the yield was 28g. (98%). A small sample was further recrystallized from the same solvent to give 25-oxonorcholesteryl acetate (II), m.p. 138–139° [Dauben & Bradlow (1950) give m.p. 138·5–139·5°]. It had R_F (system *B*) 0·49 (25-oxonorcholesterol

has R_F 0·68). Both compounds gave yellow spots when sprayed with Brady's reagent while adsorbed on chromatoplates.

A solution of this acetylated ketone (27 g.) in 5% (v/v) methanol in ethanol (800 ml.) was cooled to 10° and treated with a solution of $NaBH_4$ (2·7 g.) in ethanol–water (1:1, v/v) (2 ml.). The solution was rapidly stirred at room temperature for 0·5 hr. The reaction mixture was poured into dil. HCl (500 ml.) and the ethanol evaporated at 40° *in vacuo*. The mixture was then extracted with ether (3 × 500 ml.). The combined ethereal extracts were washed with water (3 × 100 ml.), dried over $MgSO_4$ and then evaporated, yielding a white solid; the yield was 24 g. (85%). Recrystallization from ethanol–water gave (±)-25-hydroxynorcholesteryl acetate (III) as plates, m.p. 116–120°. Further recrystallization of a small sample raised the m.p. to 120–122°. It had R_F (system *C*) 0·51 (25-oxonorcholesteryl acetate has R_F 0·63) and $[\alpha]_{20}^{D} -17·4 \pm 1°$ (*c* 2 in chloroform).

A solution of this compound (20 g.) in sodium-dried ether (500 ml.) was cooled to −80°. Pyridine (3·8 ml.) was added, followed by the dropwise addition of freshly prepared thionyl bromide (4·1 ml.).

The solution was stirred and allowed to reach room temperature over 1 hr. A white precipitate of pyridine hydrobromide separated. Benzene (500 ml.) was added and the ether was removed *in vacuo*. The reaction mixture was heated under reflux for 3 hr. and filtered after cooling. The precipitate was washed with ether and the washings were added to the benzene solution. The solvents were then removed *in vacuo* at 50° to give a pale-yellow solid; the yield was 20 g. (88%). Recrystallization from light petroleum (b.p. 40–60°) gave needles, m.p. 105–107°. Recrystallization of a small sample gave (±)-25-*bromonorcholesteryl acetate* (IV) as needles, m.p. 106–108° (Found: Br, 16·9. $C_{28}H_{45}BrO_2$ requires Br, 16·2%). It had R_F (system *C*) 0·63 (25-hydroxynorcholesteryl acetate has R_F 0·27) and $[\alpha]_{D}^{20} -16·8 \pm 1°$ (*c* 2 in chloroform).

A solution of the bromoacetate (7 g.) in ether (100 ml.) was treated with a solution of methylmagnesium iodide (2·5 equiv.) in ether. The mixture was stirred at room temperature for 1 hr. Water was added to destroy the excess of the Grignard reagent. The ethereal layer was treated with dil. HCl and washed with dil. Na_2CO_3 and then water. The ethereal solution was dried over $MgSO_4$ and the ether was evaporated to give a pale-yellow solid; the yield was 5·8 g. (90%). Recrystallization of the latter from light petroleum (b.p. 40–60°) gave needles, m.p. 127–129°. Further recrystallization of a sample from the same solvent gave (±)-25-*bromonorcholesterol* (V) as needles, m.p. 128–130°. It had R_F (system *B*) 0·46 [(±)-25-bromonorcholesteryl acetate has R_F 0·645].

The n.m.r. spectrum (Table 1) indicated that the bromine atom was at C-25, since the spectrum showed a single proton (i.e. secondary bromide) with an adjacent methyl group in addition to the C-20-proton interactions with the C-21-methyl group.

A solution of the bromonorcholesterol (1·8 g.) in distilled 2,3-dihydropyran (6·5 ml.) was treated with conc. HCl (2 drops) and the mixture shaken for 10 min. and left to stand overnight. Ether (50 ml.) was added and the solution was treated with solid KOH (about 3 g.) for 10 min., when the solution was filtered. The ether was evaporated *in vacuo* to give a white solid, which on recrystallization from di-isopropyl ether gave (±)-3β-*tetrahydropyranyloxy*-25-*bromonorcholest*-5-*ene* (VI) as beautiful needles, m.p. 156–158°. It had R_F (system *B*) 0·70 [(±)-25-bromonorcholesterol has R_F 0·46] (Found: C, 70·0; H, 9·83; Br, 15·8; $C_{31}H_{51}BrO_2$ requires C, 69·5; H, 9·6; Br, 14·9%).

A solution of bromonorcholesterol (1·0 g.) in dimethyl sulphoxide (20 ml.) was treated with dried KCN (1·0 g.). The reaction mixture was heated under reflux, under N_2, for 3·5 hr. The solution was cooled and poured into water (200 ml.). The precipitate that formed was filtered off and washed with water until the washings gave no precipitate with aqueous $AgNO_3$ solution. The precipitate was dried *in vacuo* for several hours and recrystallized from light petroleum (b.p. 40–60°), giving (±)-25-*cyanonorcholesterol* (VII) as needles, m.p. 110–117°. These crystals proved to be inseparable from small amounts of the starting material and were also contaminated with a by-product that had the same R_F as (±)-25-hydroxynorcholesterol, prepared by reduction of 25-oxonorcholesterol with $NaBH_4$ in ethanol. The R_F values of compounds in the mixture were 0·455, 0·56 and 0·62 [(±)-25-bromonorcholesterol has R_F 0·62; (±)-25-hydroxynorcholesterol has R_F 0·45]. Further purification did not prove necessary, however.

A solution of the product of the above reaction (1·0 g.) in ethanol (10 ml.) was treated with KOH (500 mg.) and heated under reflux, under N_2, for 18 hr. The reaction mixture was cooled and poured into dil. H_2SO_4 (100 ml.). The insoluble precipitate that formed was washed with water and dried. Recrystallization of the solid from acetic acid–water gave (±)-3β-hydroxycholest-5-en-26-oic acid (VIII) as plates, m.p. 176–178° [Kučera & Šorm (1958) reported plates, m.p. 173–175°]. It had R_F (system *A*) 0·17 (a sample kindly supplied by Professor Šorm had R_F 0·17).

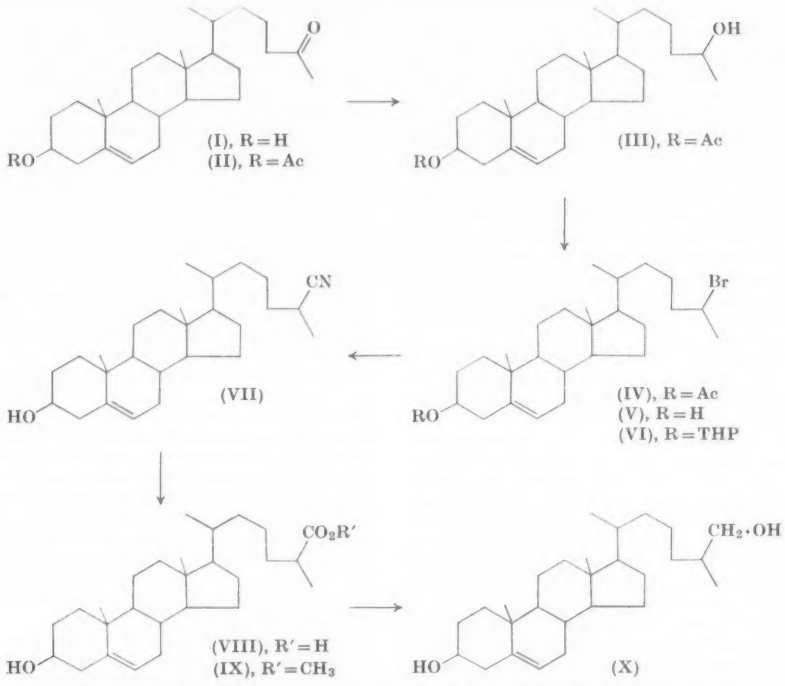
Preparation of (±)-26-*hydroxycholesterol* (*X*) (*Scheme* 1). A solution of the hydroxycholestenoic acid (0·5 g.) in ether

Table 1. *Proton resonances of* 25-*bromonorcholesterol*

τ values of groups in deuterochloroform

CH_3 (C-18)	CH_3 (C-21)	CH_3 (C-19)	CH_3 (C-27)	CH·OH (C-3)	CH_2·CHBr·CH_3 (C-25)	C·CH·CH·CH_2 (C-6)
9·31	9·08 (doublet) (*J*, 6 cyc./sec.)	8·99	8·295 (doublet) (*J*, 6·6 cyc./sec.)	8·1*	5·845 (sextet) (J_{total}, 31·8 cyc./sec.)	4·635 (ill-defined doublet) (*J*, 3 cyc./sec.)

* This is the only peak quenched on the addition of deuterium oxide.



Scheme 1. Synthesis of 26-hydroxycholesterol. Ac, Acetyl; THP, tetrahydropyranyloxy.

(10 ml.) was treated with an excess of an ethereal solution of diazomethane and left to stand for 20 min. Dilute aq. acetic acid was added and the ethereal layer was washed with water and dried over $MgSO_4$. The ether was evaporated to give a white solid. Recrystallization from methanol gave methyl (±)-3β-hydroxycholest-5-en-26-oate (IX) as needles, m.p. 100–103° [Kučera & Šorm (1958) reported needles, m.p. 102–103°]. It had R_F (system *B*) 0·52 (an authentic sample kindly supplied by Professor Šorm had R_F 0·52).

A solution of this ester (500 mg.) in sodium-dried ether (10 ml.) was treated with an excess of an ethereal solution of $LiAlH_4$ (about 1 g.) and the mixture heated under reflux for 0·5 hr. The excess of $LiAlH_4$ was destroyed by the careful addition of acetone diluted with ether, then acetone alone and finally dil. HCl. The mixture was extracted with ether (3 × 50 ml.) and the ethereal extracts were combined, washed with water and dried over $MgSO_4$. The ether was evaporated to give a white solid, which on recrystallization from light petroleum (b.p. 60–80°)–ethyl acetate gave (±)-26-*hydroxycholesterol* (X) as needles, m.p. 168–173° [Scheer, Thompson & Mosettig (1956) reported m.p. 177–178°]. It had R_F (system *B*) 0·35 (methyl hydroxycholestenoate has R_F 0·52; two samples of authentic 26-hydroxycholesterol kindly supplied by Dr Y. Sato and Dr E. Staple respectively had R_F 0·35).

Preparation of (±)-3β-*hydroxy*[26-^{14}C]*cholest-5-en-25-oic acid*. A solution of (±)-25-bromonorcholesterol (70 mg.) (prepared as above) in dimethyl sulphoxide (7 ml.) was treated with $Na^{14}CN$ (1·5 mg.) and the solution was heated under reflux in an atmosphere of N_2 for 6 hr. The solution was then cooled and poured into water (7 ml.) and extracted with ether (3 × 5 ml.). The ethereal extracts were combined and the ether was evaporated in a stream of N_2. The gummy residue was dissolved in ethanol (4 ml.), and aq. 3N-KOH (2 ml.) was added. The solution was heated under reflux in N_2 for 18 hr. The reaction mixture was poured into water (5 ml.) and extracted with light petroleum (b.p. 60–80°) (2 × 5 ml.). The aqueous phase was acidified with 4N-H_2SO_4 and extracted with ether (4 × 5 ml.). The ethereal extracts were combined and evaporated in a stream of N_2 to about 200 μl. The solution was then adsorbed on silica gel H plates (20 cm. × 20 cm.) and eluted with ethyl acetate. Bands corresponding to spots of authentic (±)-3β-hydroxycholest-5-en-26-oic acid were removed and extracted with methanol. This process was repeated twice. Finally the methanol solution was evaporated and the yield of radioactive acid from $Na^{14}CN$ determined to be 150 μc (about 30%).

Preparation of (±)-26-*hydroxy*[26-^{14}C]*cholesterol*. A solution of (±)-3β-hydroxy[26-^{14}C]cholest-5-en-26-oic acid (0·09 mc) in ether (1 ml.) was treated with excess of ethereal

diazomethane and the solution left to stand at room temperature for 0·5 hr. The solution was then evaporated at 10° in a stream of N_2, when excess of diazomethane was removed together with the ether. A solution of $LiAlH_4$ (10 mg.) in dry ether (1 ml.) was then added and the reaction mixture heated under reflux for 0·5 hr. The excess of $LiAlH_4$ was destroyed by adding acetone and the resulting suspension extracted successively with 2N-H_2SO_4, 2M-Na_2CO_3 and finally water. The ethereal layer was evaporated to 50 μl. and (±)-26-hydroxy[26-^{14}C]cholesterol was isolated by thin-layer chromatography, as described above, with 20 cm. × 20 cm. plates coated with silica gel H and developed with system *B*.

Preparation of 7α-hydroxycholesterol. Cholesteryl benzoate (2·5 g.) was converted into 7α-hydroxycholesteryl benzoate by using *tert.*-butyl perbenzoate and CuBr in acetic acid, as described by Starka (1962).

The crude benzoate was hydrolysed with excess of $LiAlH_4$ in dry ether for 1 hr. at room temperature. The excess of $LiAlH_4$ was cautiously destroyed by acetone. The resulting suspension was washed successively with dil. H_2SO_4, dil. Na_2CO_3 and water. The ethereal solution was dried over $MgSO_4$ and the ether was evaporated to give a yellow gum (2 g.), which was adsorbed on alumina (activity III on the Brockman scale) (100 g.) and eluted with benzene (3500 ml.), ether (1300 ml.) and methanol–ether (1:19, v/v) (300 ml.). Fractions of volume 100 ml. were collected and a small portion of each fraction was examined by thin-layer chromatography on silica gel H plates developed with system *B* (Table 2).

Fractions 37–47 were recrystallized from methanol to give 7α-hydroxycholesterol as needles, m.p. 180–182° (literature m.p. 185°). It had R_F 0·57 (7β-hydroxycholesterol has R_F 0·63). The yield was about 1 g. (50%).

Preparation of 7α-hydroxy[26-^{14}C]*cholesterol.* [26-^{14}C]-Cholesterol (1·9 mg.) was dissolved in pyridine (0·5 ml.) and cooled to 0°. The solution was treated with cold benzoyl chloride (10 μl.) and left at room temperature for 16 hr. Ice-cold water (1 ml.) and 2N-H_2SO_4 (1 ml.) were added and the solution was extracted with ether (3 × 2 ml.). The ethereal extracts were combined and washed with water (2 × 1 ml.). The ether was evaporated in a stream of N_2. Acetic acid (0·5 ml.) and CuBr (1 mg.) were added and the solution was heated under reflux, under N_2, by immersion in an oil bath at 120°. A solution of *tert.*-butyl perbenzoate (10 μl.) in acetic acid (0·5 ml.) was added over 15 min. The solution was further heated under reflux at 125–130° for 10 min. and then cooled. Benzene (2 ml.) was added. The solution was extracted successively with water (1 × 2 ml.), 5% (w/v) Na_2CO_3 solution (1 × 2 ml.) and water (1 × 2 ml.). Ether (2 ml.) was added and the solution treated with excess of $LiAlH_4$. After standing for 1 hr. at room temperature the excess of $LiAlH_4$ was destroyed with acetone and the ethereal suspension was extracted with water (1 ml.), dil. H_2SO_4 (1 × 2 ml.) and finally again with water (1 ml.). The ethereal solution was evaporated in a stream of N_2 to a small volume (50 μl.). The latter solution was adsorbed on silica plates (20 cm. × 20 cm.) and developed with system *B*. Authentic non-radioactive 7α-hydroxycholesterol was also chromatographed at the side and in the centre of these plates. Areas containing the radioactive 7β-hydroxycholesterol (R_F 0·57) [revealed on spraying only the non-radioactive areas (R_F 0·57) with $SbCl_3$] were scraped off and extracted with methanol–ether (1:19, v/v). The chromatographic purification was repeated to constant specific activity. Three such purifications gave 7α-hydroxy-[26-^{14}C]cholesterol in 12–20% yield; 50% of the [26-^{14}C]-cholesterol was recovered unchanged.

Preparation of 7-dehydrocholesterol. 7-Dehydrocholesterol was prepared according to the method of Hunzicker & Müllner (1958). Cholesteryl benzoate was used as the starting material and the second double bond was introduced by using 2,3-dibromo-5,5-dimethylhydantoin (bromantin) and trimethyl phosphite. The benzoyl group was removed reductively with $LiAlH_4$ instead of by KOH hydrolysis (used by Hunzicker & Müllner, 1958). The product was purified by chromatography on alumina and recrystallized from acetone to give 7-dehydrocholesterol as needles, m.p. 145° (literature m.p. 145–147°). It had R_F (system *B*) 0·66 and $\lambda_{max.}$ in methanol at 273, 283 and 295 mμ (ϵ 10800, 11500 and 6500 respectively).

Preparation of 7-dehydro[26-^{14}C]*cholesterol.* [26-^{14}C]-Cholesterol (1·5 mg., 0·1 mc) was benzoylated, as described above in the preparation of 7α-hydroxycholesterol. The purified product was dissolved in heptane (b.p. 100°) and treated with 2,3-dibromo-5,5-dimethylhydantoin (1 mg.) in light petroleum (b.p. 100–120°) under N_2. The solution was heated under reflux at 100–120° with stirring for 10 min. The reaction mixture was cooled and centrifuged at 300*g* for 5 min. to remove precipitated 5,5-dimethylhydantoin. The supernatant was removed and the precipitate was washed with cold light petroleum (b.p. 100–120°) and further centrifuged until the supernatant was clear. The combined supernatants were evaporated to dryness and dissolved in dry xylene (3 ml.). This solution was added to a refluxing solution of trimethyl phosphite (10 μl.) in xylene (1 ml.). The reaction mixture was further heated under reflux for 1·5 hr. at 125° and subsequently cooled. The solution was evaporated at 80° *in vacuo* to dryness. Dry ether was added and the solution was treated with $LiAlH_4$ (about 10 mg.) and

Table 2. *Purification of 7α-hydroxycholesterol*

Fraction no.	Eluent	Volume (ml.)	Colour with $SbCl_3$	m.p.	R_F (system *B*)
1–20	Benzene	2000	Blue		0·83
21–27	Benzene	700	—		—
28–33	Benzene	600	Pink		0·74
34–36	Ether	300	Blue–pink		0·60–0·74
37–47	Ether	1000	Blue	173–180°	0·57
48–51	Methanol–ether (1:19, v/v)	300	Blue	238–242°	0·30

left at room temperature for 12hr. Acetone was cautiously added and the solution extracted with dil. HCl until clear. The ethereal solution was washed with water and evaporated to dryness under N_2, and the solid was purified by thin-layer chromatography as described above to give 7-dehydro-[26-^{14}C]cholesterol (the yield was 10% from [26-^{14}C]-cholesterol). It had R_F (system *B*) 0·66.

A sample of the above compound was added to 5mg. of the pure 7-dehydrocholesterol and the mixture was examined by thin-layer chromatography. In one series of experiments the plate was examined under u.v. light and the fluorescent region was removed, extracted with ether and made up to a known volume with ethanol after the ether had been removed under N_2. Portions of this solution were assayed for ^{14}C by liquid-scintillation counting; portions were also assayed spectrophotometrically for 7-dehydrocholesterol by measuring the extinction at 272, 282 and 294mμ. Three successive purifications by thin-layer chromatography gave material of constant specific activity.

Cholesteryl 3β-O-monosulphate. Sodium [26-^{14}C]cholesteryl 3β-*O*-monosulphate (24mc/m-mole) was synthesized from [26-^{14}C]cholesterol and pyridine–SO_3 (Roy, 1956) and freed from cholesterol by thin-layer chromatography on silica with ethyl acetate–hexane (3:7, v/v). The immobile cholesteryl sulphate was then eluted and purified by thin-layer chromatography on alumina with methanol–water (9:1, v/v). Solvolysis with trichloroacetic acid and dioxan regenerated only cholesterol.

RESULTS

Oxidation of hydroxycholesterols by liver preparations

Oxidation of the terminal isopropyl group. Table 3 shows that the mitochondrial fraction of mouse livers contained the bulk of the sterol-oxidase activity and that added 26-hydroxy-[26-^{14}C]cholesterol was oxidized to $^{14}CO_2$ much more readily than added 7α-hydroxy[26-^{14}C]cholesterol, which in turn was oxidized considerably more rapidly than [26-^{14}C]cholesterol or 7-dehydro-[26-^{14}C]cholesterol.

Fig. 1 compares the relative rates of oxidation of 7α- and 26-hydroxycholesterol by mouse-liver mitochondria. Essentially the same difference in rates of oxidation of these two hydroxycholesterols was observed with guinea-pig mitochondrial preparations. The strain of guinea pig that we used (bred in this Laboratory) secretes chenodeoxycholic acid as the principal bile acid, which was identified by thin-layer chromatography (Hofmann, 1962) and colour reaction with ferric chloride and sulphuric acid (Anthony & Beher, 1964).

Thus although the mouse and the guinea pig secrete different bile salts (Haslewood, 1964) there is no obvious difference between liver mitochondrial preparations of these two mammals with regard to

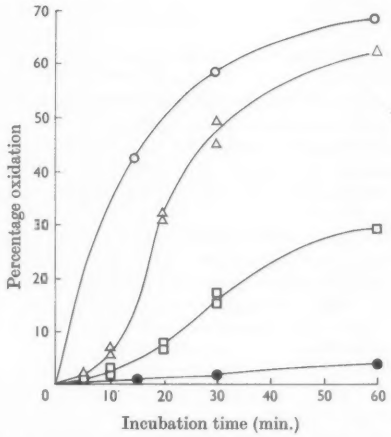


Fig. 1. Comparative rates of oxidation of (±)-3β-hydroxycholest-5-en-26-oic acid (○), (±)-26-hydroxycholesterol (△), 7α-hydroxycholesterol (□) and cholesterol (●), determined as the yield of $^{14}CO_2$ by fortified mouse-liver mitochondria. Experimental details are given in the text.

Table 3. *Relative oxidation of* [26-^{14}C]*sterols by mouse-liver preparations*

Supernatant fraction was present in all incubations. The final pH was 8·2 at 37°. The percentage oxidation was computed as $^{14}CO_2$ evolved × 100/[^{14}C]sterol substrate.

	Percentage oxidation				
Liver fraction	Cholesterol	7-Dehydro-cholesterol	7α-Hydroxy-cholesterol	26-Hydroxy-cholesterol	Cholesteryl sulphate
Mitochondrial	4·0	6·0	18·5	60	0·2
Microsomal	0·3	0·2	5·5	1·0	0·01
Mitochondrial plus microsomal	0·2	0·3	2·1	18·7	0·1
Incubation time (hr.)	1	1	0·5	0·5	3
[^{14}C]Substrate added/ incubation (μc)	0·025	0·05	0·01	0·01	0·05

Table 4. *Oxidation of hydroxycholesterols and 3β-hydroxycholest-5-en-26-oic acid to carbon dioxide and steroid acids by mouse, English toad and South American cayman liver mitochondrial preparations*

Results are given as percentage conversions of [^{14}C]substrate (0·05 μc) into $^{14}CO_2$ or [^{14}C]steroid acids on incubating fortified mitochondrial preparations from equal weights of livers with homologous SF for 1 hr. at 37° in tris hydrochloride buffer, pH 8·2. N.D., Not determined.

Substrate	Product measured	Percentage conversion		
		Mouse	Cayman	Toad
26-Hydroxycholesterol	CO_2	60	1	5
7α-Hydroxycholesterol	CO_2	19	0·6	0·7
3β-Hydroxycholest-5-en-26-oic acid	CO_2	80	1	10
Cholesterol	CO_2	4	0	0
26-Hydroxycholesterol	Acids	N.D.	47	50
7α-Hydroxycholesterol	Acids	N.D.	37	10
Cholesterol	Acids	N.D.	31	43

their ability to oxidize these two hydroxycholesterols.

Rat-heart, -kidney and -spleen homogenates did not yield significant amounts of $^{14}CO_2$ when incubated with 26-hydroxy[26-^{14}C]cholesterol under conditions in which they appreciably oxidized [2-^{14}C]octanoic acid and [2-^{14}C]propionic acid to $^{14}CO_2$.

The supernatant fraction (SF) that is required for optimum cholesterol oxidation by rat- and mouse-liver preparations (Whitehouse, Staple and Gurin, 1961) stimulated the oxidation of both 7α- and 26-hydroxycholesterol by mouse-liver mitochondria.

Since the percentage yield of $^{14}CO_2$ from incubations of 26-hydroxy[26-^{14}C]cholesterol with mouse-liver mitochondria was usually above 50% after 1 hr. (e.g. 72% at 2 hr. in one experiment), there was evidently no ultimate discrimination between the two optical isomers of 26-hydroxycholesterol (due to the asymmetric C-25) by the mitochondrial sterol oxidase (cf. Berséus, 1965).

The mitochondrial fractions from toad and cayman livers were incubated with the hydroxycholesterols and other sterols, and both the $^{14}CO_2$ and the [^{14}C]steroid acids formed therefrom were determined. Table 4 compares the results obtained from these experiments and from parallel experiments with mouse-liver mitochondria. Both these non-mammalian liver preparations oxidized 26-hydroxycholesterol to both carbon dioxide and steroid acid(s) more readily than they oxidized 7α-hydroxycholesterol. This acid fraction, assayed after thin-layer chromatography, did not include propionic acid, a potential metabolite of steroid acids.

Oxidation of related sterols. A racemic mixture of 3β-hydroxy[26-^{14}C]cholest-5-en-26-oic acid was very rapidly oxidized to $^{14}CO_2$ by mouse-liver mitochondria, the initial rate being at least three times that of 26-hydroxycholesterol over the first 15 min. (Fig. 1). The relative yield of $^{14}CO_2$ was always greater than 50% after 20 min. incubation, indicating that there was again no ultimate discrimination by the mitochondrial oxidase between the C-25 optical isomers.

Cayman-liver mitochondria did not significantly oxidize the carboxylic acid group of this acid to $^{14}CO_2$ (Table 4), but toad-liver mitochondria had some definite capacity to do so. This finding confirms what is known about the distribution of the side-chain-cleaving enzymes (Whitehouse, Cottrell, Briggs & Staple, 1962; Haslewood, 1964). The cayman-liver mitochondria readily oxidized [2-^{14}C]propionic acid to $^{14}CO_2$.

Cholesteryl sulphate has been implicated as a biological precursor of adrenal steroids (Roberts, Bandi, Calvin, Drucker & Lieberman, 1964). [26-^{14}C]Cholesteryl 3-*O*-monosulphate was not significantly oxidized by mouse-liver mitochondria (Table 3), suggesting that it is not a precursor for bile acid formation.

26-Hydroxycholesterol dehydrogenase in mammalian liver. Table 5 shows the results of incubating 26-hydroxy[26-^{14}C]cholesterol with various fractions isolated from rat livers and determining the [26-^{14}C]steroid acid formed from the incubation mixture. A requirement for NAD was shown; in similar concentrations, NADP only partially substituted for NAD in this respect. Pretreatment of extracted sterols with moist silver oxide, before separation of the steroid acids by thin-layer chromatography, did not increase the yield of the ^{14}C-labelled acid fraction, indicating that the 26-aldehyde was not present in significant quantity in the incubation mixtures.

A comparison of the percentage conversion into the steroid acid (predominantly 3β-hydroxycholest-

Table 5. *Subcellular distribution and properties of rat-liver 26-hydroxycholesterol dehydrogenase*

The ^{14}C-labelled acid formed was assayed after thin-layer chromatography and computed as percentage of ^{14}C-labelled acid × 100/[^{14}C]sterol substrate (0·05 μc).

Liver fraction	Cofactor added	Acid formed after 1 hr. (%)	Protein (mg./incubation)
Microsomal plus supernatant	NAD	26	170
Mitochondrial particulate	NAD	9	250
Soluble mitochondrial extract	NAD	54	90
	None	8	90
	NADP	35	90
'Killed' at zero time with acid	NAD	6	90

5-en-26-oic acid) per mg. of protein for each fraction showed that the particulate mitochondrial and microsomal fractions were not very efficient in oxidizing 26-hydroxycholesterol. On the other hand, the soluble fraction extracted from washed mitochondria converted at least 50% of the substrate into the 3β-hydroxycholest-5-en-26-oic acid within 1 hr. These mitochondrial extracts also oxidized ethanol. However, preparations of highly purified horse-liver alcohol dehydrogenase failed to oxidize 26-hydroxycholesterol.

pH *optima for sterol oxidation.* The optimum pH for $^{14}CO_2$ formation from each of these various [26-^{14}C]sterols and from [2-^{14}C]propionic acid by mouse-liver mitochondria was determined by using tris hydrochloride buffers in the pH range 7·0–9·0 (pH was measured at 37°). The following pH optima were found: for cholesterol, 8·3; for 7α-hydroxycholesterol, 8·5; for 26-hydroxycholesterol, 8·1; for 3β-hydroxycholest-5-en-26-oic acid, 7·7; for propionic acid, 7·3.

The pH optimum for the oxidation of 26-hydroxycholesterol to 3β-hydroxycholest-5-en-26-oic acid was approx. 8·1 in tris hydrochloride buffer.

DISCUSSION

Preparation of 3β-hydroxy[26-^{14}C]*cholest-5-en-26-oic acid and 26-hydroxy*[26-^{14}C]*cholesterol.* Previously described methods for the preparation of 26-hydroxycholesterol have involved the degradation of sapogenins (Scheer *et al.* 1956; E. Staple, unpublished work) and are not feasible for introducing a ^{14}C label specifically in one of the terminal three carbon atoms of the sterol. Various syntheses were therefore explored based on 25-oxo-(27)-norcholesterol as a starting material. The synthesis of hydroxycholestenoic acid from 25-oxonorcholesterol described by Kučera & Šorm (1958), involving cyanhydrin formation, gives rather poor yields of the acid and was not considered practicable on a micro-scale.

An alternative route from 25-oxonorcholesterol, outlined in Scheme 1, was therefore developed, involving the following conventional procedures. 25-Oxonorcholesteryl acetate was converted into 3β-acetoxy-25-hydroxynorcholest-5-ene by controlled reduction with sodium borohydride at room temperature. This alcohol was brominated with thionyl bromide (1·1 moles) and pyridine (1·0 mole) and the acetyl group then removed by treatment with a solution of methylmagnesium iodide in ether. The resulting 25-bromonorcholesterol was converted into the cyanide (^{14}C-labelled in the radioactive synthesis) with potassium cyanide or sodium cyanide in dimethyl sulphoxide, and the cyanide hydrolysed with potassium hydroxide in ethanol to give the required 3β-hydroxycholest-5-en-26-oic acid, which was readily separated from neutral material by extraction with alkali. Treatment of this acid with diazomethane in ether, before reduction with lithium aluminium hydride, improved the yield of 26-hydroxycholesterol: the latter compound was identical with authentic samples prepared by the late Dr Mosettig by another route.

While attempting to prepare 3β-acetoxy-25-bromonorcholest-5-ene it was found that phosphorus tribromide would not react smoothly with the 25-ol and gave resinous products from which was isolated a compound, m.p. 145–147°, which on analysis contained no bromine; this compound has not been further investigated.

It was found that removal of the 3-acetoxy group was necessary before introducing the cyanide group, since potassium cyanide reacted with 3β-acetoxy-25-bromonorcholest-5-ene to give a compound, m.p. 130–132°, which had a mobility on thin-layer

plates identical with that of 3β-hydroxynorcholest-5-ene, m.p. 165–167°; this compound has not been further investigated.

Preparation of 7α-hydroxy[26-^{14}C]*cholesterol and 7-dehydro*[26-^{14}C]*cholesterol.* Routes to 7α-hydroxycholesterol include: (*a*) allylic bromination of cholesterol with *N*-bromosuccinimide (Bide, Henbest, Jones, Peevers & Wilkinson, 1948; Bernstein, Binovi, Dorfman, Sax & Subbarow, 1949); (*b*) photo-oxidation of cholesterol to the 5α-hydroperoxide and subsequent rearrangement to the 7α-hydroperoxide followed by reduction of the hydroperoxide to the alcohol (Schenck, Gollnick & Neumüller, 1957; Lythgoe & Trippett, 1959); (*c*) oxidation of cholesteryl benzoate by *tert.*-butyl perbenzoate in the presence of cuprous bromide and acetic acid followed by removal of the benzoyl group (Starka, 1962).

This last method was found to be the most suited to adaptation on the micro-scale. The only modification for micro-synthesis was in the final hydrolysis of the benzoate, achieved reductively with lithium aluminium hydride in ether, giving adequate yields of 7α-hydroxycholesterol. Starka (1962) also described the preparation of 7-dehydrocholesterol from 7α-hydroxycholesterol by an acid-catalysed elimination of water with hydrochloric acid in methanol. This reaction was not successful on the micro-scale.

7-Dehydro[26-^{14}C]cholesterol was obtained by another route. Among the final products, a compound was detected (by thin-layer chromatography) that did not appear to be either 7α- or 7β-hydroxycholesterol but gave a vivid blue colour with antimony trichloride; this unknown compound had no absorption maximum in the u.v. region between 250 and 300mμ and had a much higher R_F than either 7α- or 7β-hydroxycholesterol.

Mitochondrial oxidation of hydroxycholesterols. Recent work suggests that the hydroxylation of the cholesterol nucleus, which probably precedes cleavage of the side chain in bile acid formation (Danielsson, 1963), takes place in microsomes (Mendelsohn & Staple, 1963; Mitton & Boyd, 1965; Danielsson, 1960; Berséus, 1965). 26-Hydroxylation of sterols appears to be confined to mitochondria (Fredrickson & Ono, 1956; Danielsson, 1961; Suld, Staple & Gurin, 1961). 26-Hydroxycholesterol may therefore not be a normal intermediate in bile acid formation from cholesterol. This present study does, however, indicate that mammalian liver mitochondria can rapidly oxidize a 26-hydroxysterol and that oxidative degradation of the sterol side chain may be rather non-specific with regard to substrate, as suggested by Bergström (1963), any apparent specificity in cholanic acid formation probably being determined by the nature of the microsomal hydroxylases.

If the 26-hydroxylase is really a mitochondrial enzyme, then it appears to be rather rate-limiting for the side-chain oxidation of sterols, since 7α-hydroxycholesterol was oxidized much less rapidly than 26-hydroxycholesterol by both mammalian and non-mammalian liver mitochondrial preparations.

The soluble enzyme contained in mitochondria that oxidizes 26-hydroxycholesterol to 3β-hydroxycholest-5-en-26-oic acid appears to be very similar to that described by Herman & Staple (1965), which oxidizes 3α,7α,12α,26-tetrahydroxy-5β-cholestane to the corresponding 26-carboxylic acid, although these authors did not identify the subcellular location of this enzyme. Like liver alcohol dehydrogenase (Dalziel & Dickenson, 1965), the 26-hydroxycholesterol dehydrogenase(s) can use NADP as the coenzyme in place of NAD. These two dehydrogenases are not identical however, as the purified alcohol dehydrogenase does not oxidize 26-hydroxycholesterol.

We are most grateful to Dr K. Dalziel for providing crystalline alcohol dehydrogenase and assisting with fluorimetric assays, to Dr Y. Sato, Dr E. Staple and Academician F. Šorm for providing authentic samples of 26-hydroxycholesterol, 3β-hydroxycholest-5-en-26-oic acid and its methyl ester, to Mr P. R. H. Raggatt for synthesizing cholesteryl 3β-sulphate and to the Medical Research Council for a Scholarship (for P. D. G. D.).

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The Preparation and Properties of Ficin Chemically Attached to Carboxymethylcellulose

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(*Received* 30 *July* 1965)

1. Purified ficin has been coupled to four CM-celluloses by reaction with their acid azide derivatives. Insoluble products containing 1·8–4·7 mg. of ficin/100 mg. of product and retaining 8·0–12·0% of the free enzyme's esterase activity have been obtained. 2. The amount of bound ficin in these preparations is dependent on the degree of carboxymethyl substitution of the CM-cellulose to which the ficin is attached. 3. A shift of the alkaline limb of the pH–activity curve of ficin when chemically attached to CM-cellulose has been shown. 4. Only a small loss has been observed in the enzymic activity of these products when stored at 2° for 4 months. They are more resistant than free enzyme to heat denaturation. 5. Columns of CM-cellulose–ficin have been packed. The degree of hydrolysis of perfused substrate has been measured for different flow rates through the column. 6. The properties of these derivatives have been discussed.

Water-insoluble derivatives of enzymes have been prepared in the following ways: (*a*) by chemical attachment of the enzyme to a reactive polymer (Bar-Eli & Katchalski, 1960, 1963; Cebra, Givol, Silman & Katchalski, 1961; Levin, Pecht, Goldstein & Katchalski, 1964; Mitz & Summaria, 1961; Manecke, 1962); (*b*) by physical adsorption of the enzyme to a charged polymer (Mitz, 1956; McLaren & Estermann, 1956; Barnett & Bull, 1959; Nikolaev & Mardashev, 1961; Nikolaev, 1962); (*c*) by entrapping the enzyme in the insoluble matrix of a cross-linked polymer (Bernfield & Wan, 1963); (*d*) by cross-linking of an enzyme by a bifunctional reagent (Quiocho & Richards, 1964).

The present paper describes the preparation and properties of ficin (EC 3.4.4.12) chemically attached to CM-celluloses. A preliminary account of some of this work has been given (Lilly, Money, Hornby & Crook, 1965).

MATERIALS AND METHODS

Purification of ficin. Crude ficin (250g.) (Cambrian Chemicals Ltd., London, S.E. 16) was purified by the method described by Hammond & Gutfreund (1959). A solution of the precipitate obtained from the first NaCl fractionation was exhaustively dialysed against deionized water and used in all subsequent experiments. The purified enzyme was assayed for esterase activity as described below. Protein in solution was measured by the microbiuret method of Itzhaki & Gill (1964) and by the extinction at 280mμ. For this an exhaustively dialysed solution of ficin was freeze-dried and then further dried over P_2O_5. A solution of this material gave a specific extinction coefficient of 22·6.

Preparation of p*-chloromercuribenzoate-blocked ficin.* Ficin was reduced by cysteine (British Drug Houses Ltd., Poole, Dorset), separated from the latter on a column of Sephadex G-25 (Pharmacia Fine Chemicals Ltd., Uppsala, Sweden) and then blocked with PCMB* at pH 7·0 as described by Liener (1961). No residual activity towards BAEE was detected, but on treatment with cysteine 100% of the original activity could be recovered.

Preparation of CM-cellulose–ficin. By using the method of Mitz & Summaria (1961), ficin was coupled to CM-celluloses 30 and 70 (Whatman powder) and to CM-celluloses 50 and 90 (Mann Research Laboratories Inc., New York, N.Y., U.S.A.). These materials have different degrees of carboxymethyl substitution as shown in Table 1. The acid hydrazides of each CM-cellulose were prepared in 20g. quantities and stored in a desiccator. After conversion into their respective acid azides, 10g. of each material was suspended in 50ml. of water at 1° and the suspension quickly titrated to pH 8·7. To each suspension 450ml. of 0·05M-borate buffer, pH 8·7, was added, followed immediately by 5g. of the protein dissolved in 650ml. of water. The suspensions were stirred for 60min. at 1°, after which time the products were separated by filtration and washed

* Abbreviations: PCMB, *p*-chloromercuribenzoate; BAEE, *N*-α-benzoylarginine ethyl ester hydrochloride.

successively with two 1l. quantities of mN-HCl and similar amounts of water. To ensure the removal of physically attached protein the products were further washed with 1·0M-NaCl (2×1l.), 0·5M-$NaHCO_3$ (2×500ml.), 1·0M-NaCl (1l.) and water (2×1l.). The products were resuspended in water and stored at 2°.

Determination of chemically attached protein. The protein content of the preparations was determined by hydrolysis in 6N-HCl for 42hr. at 120° followed by estimation of the amino acids in the hydrolysate by the method of Cocking & Yemm (1954). A standard curve was obtained by treating known amounts of ficin in a similar manner.

Determination of enzymic activities. The esterase activity of ficin and CM-cellulose–ficin was determined by measuring the initial rate of hydrolysis of BAEE (L. Light and Co. Ltd., Colnbrook, Bucks.) at 25° with a TTT1c automatic titrator (Radiometer, Copenhagen, Denmark). Assays were carried out at pH6·5 in a total reaction volume of 3·0ml. in the presence of 5mM-cysteine, mM-EDTA and 0·1M-NaCl.

The proteolytic activity of the enzyme and its derivatives was determined by measuring the rate of hydrolysis of casein at 25° as described by Kunitz (1946).

For both assay methods the measured activity of CM-cellulose–ficin derivatives was found to be dependent on the rate of stirring of the reaction mixture. Therefore all assays were carried out at a stirring rate which produced maximal activity under the conditions of the assays.

RESULTS

Purification of ficin. A threefold purification of the crude ficin was observed in the final product. This material had V 5–6μmoles of BAEE hydrolysed/min./mg. of protein and K_m $2·0 \times 10^{-2}$M. These results are in accordance with those reported by Holloway, Mathias & Rabin (1964) and Bernhard & Gutfreund (1956). The purified enzyme, when chromatographed at 2° on a CM-cellulose 70 column equilibrated with mM-acetate buffer, pH4·4, was eluted by a sodium chloride gradient in a similar manner to that reported by Liener (1961).

Preparation of CM-cellulose–ficin. In the results given here all the bound protein is believed to be

Table 1. *Properties of commercial carboxymethylcellulose ion-exchange resins*

The degree of carboxymethyl substitution is expressed as the number of carboxymethyl groups/glucose monomer.

CM-cellulose	Exchange capacity (m-equiv./g.)	Degree of carboxymethyl substitution
30	0·3	0·049
50	0·5	0·083
70	0·7	0·118
90	0·9	0·154

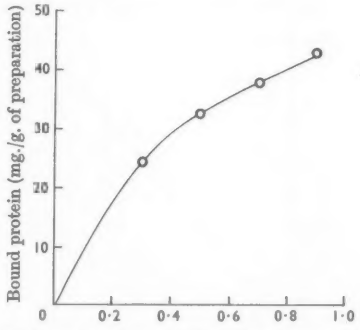

Fig. 1. Relation between the exchange capacity of CM-cellulose and its protein-binding capacity. All preparations were carried out under identical conditions and the products were subjected to the same washing procedure. The protein content of each CM-cellulose–ficin was determined by the ninhydrin method. Experimental details are given in the text.

Table 2. *Effect of protein concentration in the coupling reaction mixture on the amount and enzymic activity of ficin bound by carboxymethylcellulose*

Experimental details are given in the text.

Prep. no.	Ficin (mg./g. of CM-cellulose–azide in coupling reaction)	Ficin coupled (mg./g. of derivative)	Esterase activity (μmole of BAEE hydrolysed/min./mg. of prep.)	Esterase activity (μmole of BAEE hydrolysed/min./mg. of protein)
1	50	18	0·0135	0·80
2	100	26	0·019	0·73
3	200	30	0·021	0·69
4	300	37	0·022	0·60
5	400	43	0·025	0·58
6	500	47	0·025	0·54

chemically attached. The complete removal of the physically attached protein has been confirmed in a control experiment in which unmodified CM-cellulose replaced its corresponding acid azide in the coupling reaction mixture. In this case, when the product was subjected to the washing procedure described above, no residual protein and no esterase activity could be detected.

In coupling experiments with CM-cellulose 70 the amount of chemically attached protein was shown to be dependent on the reaction volume and the extent of the agitation. Therefore, in all comparative experiments, the coupling reactions were performed in identical vessels with the same degrees of agitation.

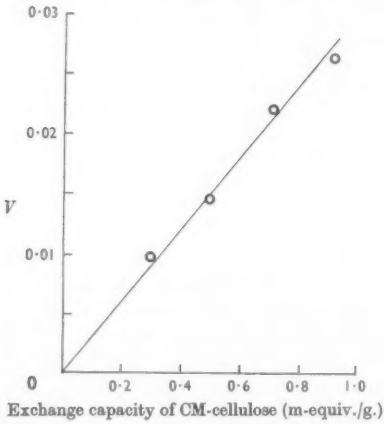

Fig. 2. Effect of the exchange capacity of the CM-cellulose on the activity of CM-cellulose–ficin preparations. The measurements of V (μmoles of BAEE hydrolysed/min./mg. of preparation) were made at 25° and pH 6·5 in the presence of 5 mM-cysteine, mM-EDTA and 0·1 M-NaCl. Experimental details are given in the text.

The effect of varying the amount of enzyme in the coupling reaction mixture, keeping all other conditions as described in the Materials and Methods section, was investigated with the acid azide of CM-cellulose 70. These results are shown in Table 2.

On reaction with identical amounts of enzyme under the same conditions the acid azides of four different CM-celluloses (see the Materials and Methods section) yielded products whose protein contents and enzymic activities were shown to be dependent on the degree of carboxymethyl substitution in the original cellulose (Figs. 1 and 2).

The effect of coupling PCMB-blocked ficin to the acid azide of CM-cellulose 70 was investigated. The amount of bound protein and its activity, after washing with cysteine, were found to be similar to those observed in a control experiment with unblocked enzyme (Table 3).

Properties of CM-cellulose–ficin. The effect of substrate concentration on the initial rates of hydrolysis of BAEE by CM-cellulose 70–ficin and CM-cellulose 90–ficin was investigated. Michaelis–Menten kinetics were shown to be obeyed and K_m 2×10^{-3}–$2{\cdot}5 \times 10^{-3}$ M was obtained for these two preparations by using the method of Lineweaver & Burk (1934) (Fig. 3). The esterase activities of preparations with CM-celluloses 30, 50, 70 and 90 were compared by assaying in a BAEE concentration that produced maximal initial velocities for each preparation. These results are given in Table 4.

The effect of pH on the initial rates of hydrolysis of BAEE by ficin and CM-cellulose–ficin preparations has been compared (Fig. 4). The displacement of the alkaline limb of the pH–activity curve with CM-cellulose 70–ficin has been observed to decrease on elevation of the ionic strength.

The measurement of the rates of hydrolysis of casein, at different pH values, by ficin and CM-cellulose 70–ficin revealed that there was no shift in the pH optimum when the enzyme was attached to the cellulose. It was therefore possible to compare

Table 3. *Coupling of ficin, pretreated with* p*-chloromercuribenzoate, with carboxymethylcellulose*

After coupling the PCMB was removed from the CM-cellulose–ficin by washing with cysteine. A control experiment was done with unblocked ficin. Experimental details are given in the text. Results for two separate experiments are shown.

Preparation	Protein content (mg. of protein/g. of prep.)	Esterase activity (μmole of BAEE hydrolysed/min./mg. of prep.)
(*a*) With PCMB–ficin		
1	46	0·025
2	49	0·026
(*b*) With unblocked ficin		
1	48	0·023
2	45	0·027

the esterase and proteolytic activities of ficin and CM-cellulose–ficin derivatives at pH 7·0 as shown in Table 5.

Stability of CM-cellulose–ficin. The effect of temperature on the stability of CM-cellulose 70–ficin and ficin was investigated by incubation of the preparations at 66° for various times (Fig. 5) and at different temperatures for periods of 25 min. (Fig. 6). In both cases samples were rapidly cooled and assayed at 25°.

A suspension of CM-cellulose–ficin in water at pH 5·0 has been stored at 2° for up to 4 months with less than 15% decrease in activity. By assaying CM-cellulose 70–ficin and ficin over a period of several weeks, both in the presence and absence of cysteine, it was shown that the insoluble enzyme was less dependent on cysteine for activation than the free enzyme (Table 6).

Preparations of CM-cellulose 30–ficin and CM-cellulose 70–ficin have been freeze-dried without any change in their enzymic activities and can be stored in this form.

Hydrolysis of BAEE by columns of CM-cellulose 70–ficin. Columns (3–5 cm. × 1·0 cm. diam.) of CM-cellulose 70–ficin have been packed and perfused with solutions of BAEE at different flow rates. At a substrate concentration of 0·3 mM-BAEE in the perfusate, extents of hydrolysis of 19–99% have been observed (M. D. Lilly, W. E. Hornby & E. M. Crook, unpublished work) for flow rates in the range 15–0·5 ml./min.

DISCUSSION

Insoluble derivatives of ficin have been prepared by coupling the enzyme to CM-celluloses. In all

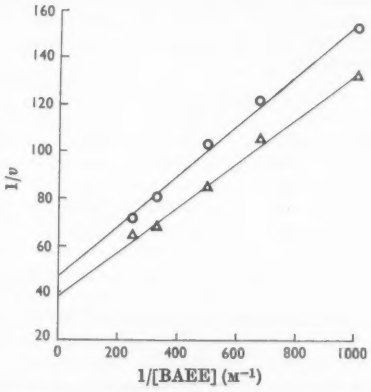

Fig. 3. Relation between substrate concentration and initial reaction velocities of the CM-cellulose 70–ficin- and CM-cellulose 90–ficin-catalysed hydrolysis of BAEE. ○, CM-cellulose 70–ficin; △, CM-cellulose 90–ficin. The initial reaction velocities (v) are expressed as μmoles of BAEE hydrolysed/min./mg. of preparation. Experimental details are given in the text.

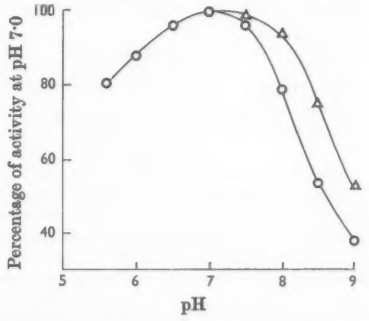

Fig. 4. Effect of pH on the initial rate of hydrolysis of BAEE by ficin (○), CM-cellulose 70–ficin (△) and CM-cellulose 90–ficin (△). Measurements were made at 25° in the presence of 5 mM-cysteine, mM-EDTA and 0·1 M-NaCl. Experimental details are given in the text. Results were corrected for CO_2 uptake by carrying out blank assays in the absence of enzyme.

Table 4. *Esterase activity of ficin chemically attached to different carboxymethylcelluloses*

Experimental details are given in the text. Esterase activity coefficient is defined as the (maximal reaction velocity/mg. of protein) × 100/(maximal reaction velocity/mg. of free ficin).

Preparation	mg. of ficin/g. of derivative	Maximal esterase activity (V) (μmole of BAEE hydrolysed/min./mg. of prep.)	Maximal esterase activity (V) (μmoles of BAEE hydrolysed/min./mg. of protein)	Esterase activity coefficient
CM-cellulose 30–ficin	24·2	0·0097	0·400	8·0
CM-cellulose 50–ficin	32·6	0·0145	0·445	8·9
CM-cellulose 70–ficin	37·6	0·0220	0·585	11·7
CM-cellulose 90–ficin	42·3	0·0263	0·620	12·4
Ficin		—	5·00	100·0

Table 5. *Proteolytic activity of ficin chemically attached to carboxymethylcelluloses*

Experimental details are given in the text. Proteolytic activity coefficient is defined as the specific proteolytic activity of bound ficin × 100/specific proteolytic activity of free ficin.

Preparation	$\Delta E_{280m\mu}$/mg. of prep.	$\Delta E_{280m\mu}$/mg. of protein	Proteolytic activity coefficient	Esterase activity coefficient/ proteolytic activity coefficient
CM-cellulose 30–ficin	0·035	0·145	4·4	1·8
CM-cellulose 50–ficin	0·044	0·150	4·5	2·0
CM-cellulose 70–ficin	0·060	0·159	4·8	2·4
CM-cellulose 90–ficin	0·063	0·174	5·2	2·4
Ficin	—	3·3	100·0	1·0

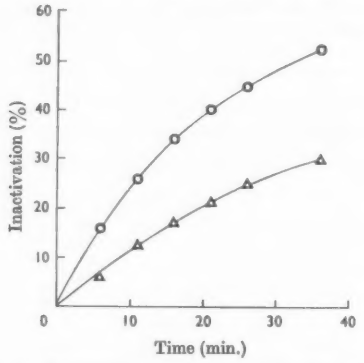

Fig. 5. Effect of temperature on the stability of ficin (○) and CM-cellulose 70–ficin (△). The preparations were incubated at 66°, samples were withdrawn at intervals and cooled in an ice bath before being assayed at 25° for esterase activity towards BAEE. Experimental details are given in the text.

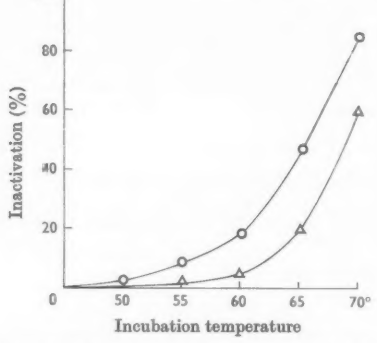

Fig. 6. Effect of different temperatures on the stability of ficin (○) and CM-cellulose 70–ficin (△). After incubation of the preparations at the different temperatures for 25 min., samples were removed, cooled in an ice bath and assayed at 25° for esterase activity towards BAEE. Experimental details are given in the text.

Table 6. *Comparison of activation of free ficin and bound ficin by cysteine*

A CM-cellulose 70–ficin suspension and a ficin solution were stored at 2°. Samples were taken at intervals and assayed for esterase activity in the presence of 5 mM-cysteine and without cysteine. Experimental details are given in the text. Activities are expressed as a percentage of the original activity.

	Ficin activity		CM-cellulose 70–ficin activity	
Time (days)	With cysteine	Without cysteine	With cysteine	Without cysteine
7	100	76	100	100
14	90	50	100	100
21	66	10	100	100

cases the protein contents of our derivatives are less than those reported by Mitz & Summaria (1961). for their insoluble derivatives. However, we have shown that the washing procedures adopted by these authors did not remove all physically attached ficin. Similar observations have been made with chymotrypsin by C. Money & E. M. Crook (unpublished work).

The bound ficin in these derivatives exhibited 9–12% of its original esterase activity and 4–5% of its proteolytic activity. The results obtained on coupling PCMB-blocked ficin to CM-cellulose 70–azide show that this loss in activity is not due to the formation of a thiol ester between the reactive thiol group of ficin and the azide group on the cellulose. In all insoluble derivatives of ficin the loss of proteolytic activity was greater than the loss of esterase activity when compared with free ficin. The greater activity of the derivatives towards smaller substrate molecules suggests some form of steric hindrance on the part of the cellulose. Such interference by the cellulose could also hinder diffusion of substrates and products to and from the active site of the enzyme. This could account for the observation that increased rates of agitation of reaction mixtures produced higher rates of

hydrolysis of casein and BAEE by CM-cellulose–ficin derivatives.

Another reason for loss in enzymic activity could be that the chemically attached enzyme is bound to the CM-cellulose in some conformation with less freedom of movement than that of the free enzyme. Such a restriction would reduce denaturation phenomena in the insoluble enzyme and an effect of this kind has been observed for the thermal denaturation of the derivatives when compared with ficin.

Values of $2{\cdot}0 \times 10^{-3}$–$2{\cdot}5 \times 10^{-3}$M for the apparent K_m values of ficin derivatives on CM-cellulose 70 and 90 are 10–12% of that observed for the free enzyme. Goldstein, Levin & Katchalski (1964) have reported a decrease in the K_m of trypsin when it is attached to a copolymer of maleic acid and ethylene. These authors have ascribed this result to the presence of areas of increased substrate concentration around the polymer due to electrostatic attraction between the positively charged substrate and the carboxyl groups of the polymer. As our system is qualitatively similar the decrease in the K_m of ficin after coupling to CM-cellulose can be explained in this way. The shift observed in the pH–activity curve of insoluble ficin is due probably to a similar phenomenon in which the carboxyl groups of the CM-cellulose produce an environment around the enzyme in which the concentration of protons is higher than that measured in the overall suspension. This explanation has been used by McLaren & Estermann (1957) to account for a similar shift in the pH–activity curve of chymotrypsin adsorbed on kaolinite and by Goldstein *et al.* (1964) for shifts observed in trypsin chemically attached to the copolymer of maleic acid and ethylene.

Although there is a loss in the enzymic activity of the bound protein in these derivatives of ficin, nevertheless, they have many practical advantages. Their increased stability, greater resistance to denaturation and reduced susceptibility to autolysis makes them suitable for use over long periods of time. By virtue of their insolubility they can be recovered from reaction media quickly and efficiently without contamination of substrate and products. With material packed in columns we have shown that it is possible to exercise great control over the rate and extent of conversion of substrate into products.

The authors express their thanks to the Whitehall Foundation Inc. for the support of this work.

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Biochem. J. (1966) **98**, 426

The Action of Neutral Hypochlorite on Epithelial Mucopolysaccharides

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(*Received* 9 *July* 1965)

1. The uptake of dilute neutral hypochlorite by epithelial mucopolysaccharides has been compared with that of proteins, polysaccharides, amino acids and sugars. The uptake has been shown to be related to the protein content of the mucopolysaccharides rather than their polysaccharide content. 2. The destruction of the components of epithelial mucopolysaccharides, certain sugars and polysaccharides after oxidation with dilute neutral hypochlorite at 0–4° has been studied. Very little destruction of the sugar components occurred and in epithelial mucopolysaccharides the only amino acid destroyed specifically was arginine. 3. Oxidation of bovine submaxillary-gland mucoprotein and ovalbumin caused very little destruction of hexosamine and no detectable liberation of this residue as a free reducing group, indicating that the *O*-seryl galactosaminide and the *N*-acyl-glycosylamine linkages reported to be present in these compounds were relatively stable to hypochlorite. 4. Depolymerization of epithelial mucopolysaccharides by neutral hypochlorite has been studied by using gel-filtration columns and compared with the depolymerization of polysaccharides and proteins under similar conditions. The polysaccharides examined were relatively resistant to oxidation whereas the proteins were extensively broken down. It is inferred that the extensive depolymerization of epithelial mucopolysaccharides by hypochlorite is related to their protein content rather than their polysaccharide content. 5. Fractionation of the products of oxidation of epithelial mucopolysaccharides by column procedures has revealed that the relative proportions of components in all fractions were similar to those in the original material. 6. Though this study does not provide unequivocal evidence from which the overall structure of this type of epithelial mucopolysaccharide may be deduced, the balance of probabilities now appears to favour a long polypeptide chain to which a large number of oligosaccharide side chains are attached via *O*-seryl and *O*-threonyl glycosidic linkages. The results, however, are also consistent with an alternating sequence of short polysaccharide and polypeptide chains and further evidence is necessary before this structure can be ruled out.

Boyland (1946) found that bovine cervical mucus was rapidly dissolved by sodium hypochlorite at pH values between 7 and 11. Other oxidizing agents such as hydrogen peroxide, potassium permanganate, potassium peroxydisulphate and potassium peroxydicarbonate were also effective in solubilizing oestrous mucus, but hypochlorites and hypobromites were the most satisfactory solvents. These could rapidly solubilize the thicker pregnancy and dioestrous mucus as well as oestrous mucus under very mild conditions. These studies were carried out on the crude mucus secretion and no attempt was made to identify the structures being attacked by this oxidation. The object of the present work was to investigate the effect of neutral hypochlorite on mucopolysaccharides isolated from crude epithelial mucus and to attempt to identify the structures being attacked. In this way it was hoped that some information could be obtained about the overall structure of the mucopolysaccharides.

It became apparent at an early stage that the polypeptide was very reactive with dilute neutral hypochlorite, whereas sugars, even when a free reducing group was present, reacted very slowly. This appeared to open a line of investigation, since

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it suggested that the amino acid residues might be destroyed preferentially, allowing isolation of the oligo- or poly-saccharide portion of the molecule. No technique that will permit this with the particular type of epithelial mucopolysaccharide under study has yet been evolved, these substances being characteristically resistant to proteolytic enzymes. Thus Pusztai & Morgan (1961) reported that, although the blood-group substances are attacked by papain and ficin to yield smaller fragments, these are still of quite high molecular weight and are not greatly reduced in nitrogen content compared with the starting material. We can confirm that treatment of cervical mucopolysaccharide with ficin or Pronase, even if repeated and prolonged, does not result in the isolation of fragments containing less than 5·5% (w/w) of nitrogen. In the event, however, our expectation of the preferential removal of peptide from epithelial mucopolysaccharides by neutral hypochlorite has not been realized and a possible reason for this failure is discussed.

EXPERIMENTAL

Materials

Amino acids, peptides and proteins. L-Arginine hydrochloride, L-glutamic acid, L-glycine and L-lysine hydrochloride were obtained from British Drug Houses Ltd., Poole, Dorset. Glycylglycine, glycylglycylglycine and ovalbumin (2× crystallized) were supplied by L. Light and Co. Ltd., Colnbrook, Bucks. Bovine serum albumin and haemoglobulin were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Gelatin was supplied by a local pharmacist. Poly-L-proline was purchased from Yeda, Rehovoth, Israel. Dr R. Aschaffenburg kindly gave us a sample of casein (total casein) and α-lactalbumin. *N*-Benzoylglycine methyl ester was generously supplied by Dr H. Gutfreund. Dioxopiperazine was prepared according to the method of Schott, Larkin, Rockland & Dunn (1947).

Carbohydrates. D-Galactose, D-glucosamine hydrochloride, D-glucose, sucrose and potato starch were obtained from British Drug Houses Ltd. *N*-Acetyl-D-glucosamine, chondroitin sulphate and larch galactan were supplied by L. Light and Co. Ltd. Dextrans of known molecular-weight range and Sephadex G-25 (medium grade) were obtained from Pharmacia, Uppsala, Sweden. For preparation of granulated agar columns Davis Standard Agar from Davis Gelatin Ltd., London, E.C. 3, was used. Samples of methyl α-D-glucopyranoside and methyl α-*N*-acetyl-D-glucosaminide were kindly supplied by Professor W. T. J. Morgan.

Epithelial mucopolysaccharides. Bovine cervical mucopolysaccharides were prepared by the method of Gibbons (1959) from pooled bovine cervical mucus plugs of pregnant animals and from pooled materials of animals at or near oestrus. The washed gels were dissolved in aqueous saturated $CaCl_2$ soln.–ethanol (9:1, v/v) and precipitated between 48 and 52% (v/v) of ethanol. The precipitate was redissolved and reprecipitated between these ethanol concentrations two further times. The behaviour of the mucopolysaccharides from pregnant animals and animals at oestrus was very similar with regard to hypochlorite oxidation, and the work in this paper refers to pregnancy material unless otherwise stated. Bovine submaxillary-gland mucoprotein was prepared according to the method of Heimer & Meyer (1956). These materials behaved as single components electrophoretically at pH5·75 and in the ultracentrifuge at pH8·5 (Gibbons, 1963). Hog gastric mucopolysaccharide was purified from commercial (British Drug Houses Ltd.) hog gastric mucoid by the phenol extraction procedure of Morgan & King (1943). This was further purified by precipitating the acidic contaminants from an aqueous 1% (w/v) solution of the mucopolysaccharide with one-fiftieth of 20% (w/v) cetyltrimethylammonium bromide. After centrifuging off the precipitate at 22620*g* for 30 min., a large excess of ethanol was added to precipitate the material in the supernatant. After standing at 0–4° overnight the precipitate was recovered by centrifuging, washed twice with ethanol, redissolved in water and reprecipitated with ethanol. The precipitate was again separated, washed twice with ethanol, redissolved in water, dialysed for several days and freeze-dried. The material thus obtained was used for the work described below.

Methods

Analytical methods. Nitrogen, hexosamine, hexose, fucose and amino acids were estimated as described by Gibbons (1959). Neuraminic acid was estimated by the thiobarbituric acid method of Warren (1959) after prior hydrolysis with 0·1 N-H_2SO_4 at 80° for 1 hr. This hydrolysis period does not liberate the neuraminic acid quantitatively from all the mucopolysaccharides studied (Gibbons, 1963) but was used as the standard condition for comparative purposes. Further, the neuraminic acid in the cervical mucopolysaccharide is entirely *N*-glycollyl, whereas the standard analytical reference substance was a crystalline laboratory preparation of the *N*-acetyl compound. The former gives only 60% of the colour yield of the latter in the thiobarbituric acid reaction. To obtain a comparison of the relative amount of nitrogen present that was released as amino nitrogen in samples where only small amounts were available, hydrolysis in 6N-HCl for 16 hr. followed by neutralization with 2N-NaOH and ninhydrin estimation according to Moore & Stein (1954) were carried out. The *N*-acetylhexosamine reaction was carried out as described by Aminoff, Morgan & Watkins (1952). Glucose was determined with glucose oxidase according to Huggett & Nixon (1957) on samples that had been hydrolysed with 0·5N-HCl for 16 hr. at 100° and subsequently neutralized with N-NaOH.

Determination of galactose by quantitative paper chromatography. Solutions of the mucopolysaccharides (3 mg.) and mannose (1 mg.) in 6·2 ml. of 0·5N-HCl were hydrolysed for 16 hr. at 100°. These were then deionized by passing them through columns (10 cm. × 1·1 cm.) of Dowex 50 (200–400 mesh) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) in the H^+ form. The eluates off these were allowed to drop straight on to columns (14 cm. × 1·1 cm.) of Amberlite IR-4B (British Drug Houses Ltd.) in the acetate form. The first 10 ml. off these Amberlite IR-4B columns was discarded and the next 25 ml. was collected and freeze-dried. The residues were dissolved in 0·05 ml. of water, and the galactose was estimated by quantitative paper chromatography according to Wilson (1959) by using mannose as an

internal standard. Standard solutions of galactose and mannose were run simultaneously.

Preparation and use of gel-filtration columns. Granulated agar-gel columns (60 cm. × 2·4 cm.) were prepared and used as described by Gibbons & Roberts (1963), 3 ml. fractions being collected. When used for estimating the depolymerization of polysaccharides and mucopolysaccharides these were calibrated with dextran fractions of known molecular-weight ranges with 0·02 M-LiCl as eluting solvent and detecting the dextrans by means of the anthrone reagent (Yemm & Willis, 1954). During these calibration runs a small amount of phenylalanine (1 mg.) was mixed with the dextran solution to correct for slight differences in column performances during different runs. In this way the elution volumes of the different dextrans could be referred to the same elution volume of phenylalanine that was detected by measuring the extinction at 215 mμ in a 1 cm. cell. For the estimation of the depolymerization of proteins, however, calibration with a mixture of sucrose (9 mg.), α-lactalbumin (5 mg.), haemoglobulin (3 mg.) and bovine serum albumin (10 mg.) with 0·3 M-NaCl for elution was used. The proteins were detected by measuring the extinction at 280 and 410 mμ in 1 cm. cells, whereas the sucrose was detected with the anthrone reagent.

Gel filtration on Sephadex G-25 columns (100 cm. × 3 cm.) was carried out with water for elution, the column being packed as recommended by Pharmacia in their brochure *Sephadex in Gel Filtration*. In the studies with gel filtration, flow rates corresponding to approx. 25 ml./hr. for a column 60 cm. × 2·4 cm. and approx. 50 ml./hr. for a column 110 cm. × 3·0 cm. were used.

Paper chromatography. This was carried out by the usual descending technique in (1) ethyl acetate–pyridine–water (2:1:2, by vol.), (2) butan-1-ol–pyridine–water (6:4:3, by vol.) or (3) 3-methylbutan-1-ol–pyridine–water–diethylamine (10:7:7:0·3, by vol.). Spray reagents used for detecting sugars were those described for the aniline hydrogen phthalate procedure of Partridge (1949) and the $AgNO_3$ method of Trevelyan, Procter & Harrison (1950). Amino acids were detected with a 0·2% solution of ninhydrin in butan-1-ol.

Oxidations with hypochlorite. The HClO was prepared by distillation of a suspension of bleaching powder (25 g.) and boric acid (50 g.) in water (750 ml.). The first 250 ml. of distillate was collected and adjusted to pH 7 with N-NaOH.

Hypochlorite uptake. Solutions of 0·06% amino acids and 0·3% sugars, polysaccharides and proteins were made up in 0·3 M-sodium phosphate buffer, pH 7. The uptake of hypochlorite by these materials was measured by mixing 1 vol. of the precooled (4°) solution of material under investigation with 2 vol. of precooled NaClO (approx. 0·05 N) and allowing them to stand at 0–4°. At intervals 1 ml. samples were withdrawn into 3 ml. of 10% (w/v) KI, 2 ml. of acetic acid was added and the I_2 liberated was titrated with 0·01 N-$Na_2S_2O_3$ with 1% (w/v) soluble starch as an indicator.

Destruction and depolymerization of material. The oxidations were carried out as described under 'Hypochlorite uptake' except that in some cases unbuffered solutions were used. In these cases it was found that, when dealing with substances that showed a low uptake of hypochlorite, the pH of the solution did not fall more than 0·5 pH unit during a 9 hr. oxidation. The destruction of components was determined by withdrawing samples at intervals, reducing the excess of hypochlorite by passing SO_2 through the solution until no colour was given with a starch–KI paper and then purging the solution with N_2 to remove excess of SO_2. Analyses were carried out on these solutions without further isolation.

Measurement of the extent of depolymerization was carried out on solutions in which the excess of hypochlorite had been reduced with SO_2 as above, then neutralized and freeze-dried. The residues (containing about 10 mg. of polysaccharide or mucopolysaccharide) were made up to 3–5 ml. with water and examined on agar columns, elution being carried out with 0·02 M-LiCl. The eluates were analysed by measuring the extinction at 215 mμ and by the anthrone reaction (Yemm & Willis, 1954) on 1 ml. samples. Uronic acid determinations (Dische, 1947) were used to assay depolymerization of chondroitin sulphate, and the orcinol reaction (Synge & Wood, 1958) was used for detecting the carbohydrate material in eluates containing ovalbumin. When the depolymerization of proteins was examined less material (about 3 mg.) was put on the columns and elution was carried out with 0·3 M-NaCl instead of 0·02 M-LiCl. The position of the peaks was determined by measuring the extinction at 280 mμ or at 215 mμ.

Fractionation of hypochlorite oxidation products. Epithelial mucopolysaccharides were oxidized with neutral hypochlorite under similar conditions to those described above. Reduction of the excess of hypochlorite at the termination of the oxidation was carried out in a number of different ways in an attempt to remove the inorganic ions from the low-molecular-weight material. The two most frequently used are given below.

(*a*) Oxidation was carried out with $Ca(ClO)_2$; to terminate the reaction the solution was acidified to pH 2 with acetic acid, freeze-dried, dissolved in water and treated with a few drops of 50% (w/v) CaI_2. The I_2 liberated was extracted with carbon tetrachloride; the aqueous layer was freeze-dried again and extracted with ethanol, and the ethanol-insoluble material was dissolved in water and fractionated on a 12% (w/w) agar column with 10% (v/v) ethanol for elution. This method was rather lengthy and gave large amounts of u.v.-absorbing material.

(*b*) It was finally concluded that the best procedure was reduction with SO_2 as described above in the section on depolymerization. The solution after freeze-drying and dissolving in water was fractionated on a 12% (w/w) agar column with 0·02 M-LiCl for elution. After analysis of the eluates at 215 mμ and by the anthrone reaction, tubes constituting part of the same peak were combined and freeze-dried. The fractions that were free of inorganic ions except for LiCl were then extracted with ethanol, dissolved in a small amount of water and freeze-dried again. The residues finally obtained were dried, weighed and analysed. Where other inorganic ions were present the residues were made up to a known volume in a standard flask and samples of this were taken for analyses. In this way the absolute amounts of each component present in the fraction could be obtained.

To fractionate further the smaller fragments that contained inorganic ions the material was put on a Sephadex G-25 column (110 cm. × 3 cm.) and eluted with water saturated with chloroform.

Deionization of material on ion-exchange columns. Before paper chromatography, solutions of galactose (2 ml.) that had been oxidized with hypochlorite and reduced with SO_2,

as described above in the section on destruction of materials, were deionized as described above in the section on determination of galactose by quantitative paper chromatography.

Reducation of the oxidation products of bovine submaxillary-gland mucoprotein and ovalbumin with sodium borohydride. Solutions of 0·1% bovine submaxillary-gland mucoprotein and 0·2% ovalbumin in 0·07 M-sodium phosphate buffer, pH 7, were oxidized with 0·44 N-NaClO at 0–4°. After 9 hr. 4 ml. samples were withdrawn, the excess of NaClO was reduced with SO_2 and the samples were then purged with N_2 to remove SO_2. These solutions were treated with 0·25 ml. of $NaHCO_3$ (40 mg./ml.) and 0·25 ml. of $NaBH_4$ (40 mg./ml.) at room temperature for 16 hr. The excess of $NaBH_4$ was then destroyed with 0·5 ml. of 0·8 N-HCl and analyses were carried out on these solutions. As controls, solutions of bovine submaxillary-gland mucoprotein and ovalbumin were mixed with a suitable amount of NaClO that had been previously reduced with SO_2 and purged with N_2. The control solutions were then reduced with $NaBH_4$ as above.

RESULTS

Hypochlorite uptake. The hypochlorite uptake by various amino acids and their derivatives at neutral pH and 0–4° is shown in Fig. 1. Although this is termed hypochlorite uptake, any chlorine bound up in chloramide formation would be released by acid and would be determined as hypochlorite. However, the conditions of the assay are such that chlorate and chlorite would not be determined (Chernyshev & Semenova, 1954), stronger acidic conditions being required to assay these. In this way hypochlorite used for oxidation rather than *N*-chlorination is determined. Fig. 1 demonstrates that the uptake of hypochlorite by free amino acids is very rapid, whereas amino acid derivatives with substituted amino and carboxyl groups such as dioxopiperazine and *N*-benzoylglycine methyl ester do not show this initial rapid uptake. There is, however, with these substituted amino acids a substantial increase in hypochlorite uptake with time of oxidation. By comparison, uptake of hypochlorite by sugars under similar conditions, shown in Fig. 2, is very much less than that of amino

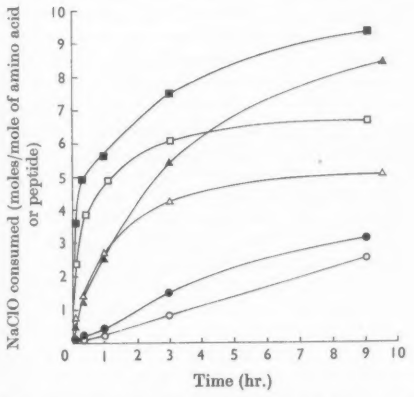


Fig. 1. Amount of NaClO consumed (moles/mole) at pH 7 and 0–4° by dioxopiperazine (○), *N*-benzoylglycine methyl ester (●), glycine (△), glycylglycine (▲), arginine (□) and glycylglycylglycine (■) plotted against time.

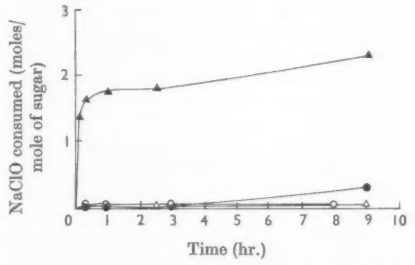


Fig. 2. Amount of NaClO consumed (moles/mole) at pH 7 and 0–4° by methyl α-*N*-acetyl-D-glucosaminide (△), galactose (○), *N*-acetylglucosamine (●) and glucosamine hydrochloride (▲) plotted against time.

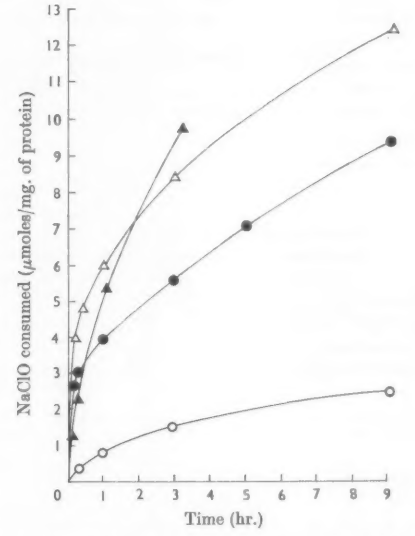


Fig. 3. Amount of NaClO consumed (μmoles/mg.) at pH 7 and 0–4° by poly-L-proline (○), ovalbumin (●), bovine serum albumin (△) and gelatin (▲) plotted against time.

acids, galactose, *N*-acetylglucosamine and methyl α-*N*-acetyl-D-glucosaminide taking up negligible amounts even after 9 hr. Glucosamine hydrochloride does show a rapid uptake of hypochlorite, due, presumably, to oxidation of the free amino groups.

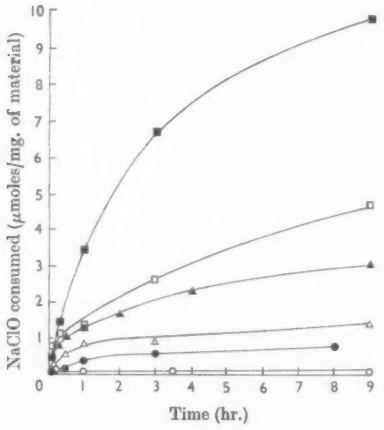


Fig. 4. Amount of NaClO consumed (μmoles/mg.) at pH 7 and 0–4° by dextran 500 (○), starch (●), chondroitin sulphate (△), hog gastric mucopolysaccharide (▲), bovine cervical mucopolysaccharide (□) and bovine submaxillary-gland mucoprotein (■) plotted against time.

In Figs. 3 and 4 a comparison is made of the hypochlorite uptake at pH 7 by proteins, polysaccharides and mucopolysaccharides. Proteins such as bovine serum albumin, casein, gelatin and ovalbumin show a rapid large uptake of hypochlorite that increases with time of oxidation. Polysaccharides such as dextran 500 and potato starch, however, show very little consumption of of hypochlorite under the conditions used. Chondroitin sulphate takes up slightly more hypochlorite, but this is still very much less than the amount taken up by the epithelial mucopolysaccharides such as bovine cervical mucopolysaccharide and hog gastric mucopolysaccharide. These show an uptake intermediate between that of proteins and the small uptake of polysaccharides. The amount of hypochlorite consumed appears to depend on their protein content, since bovine submaxillary-gland mucoprotein with its protein content of about 60% takes up a greater amount than the epithelial mucopolysaccharides of the blood-group-substance type, which contain 15–25% of amino acid residues.

Poly-L-proline takes up relatively small amounts of hypochlorite even after 9 hr. of oxidation.

Destruction of components by hypochlorite. In Table 1 the extent of destruction of galactose, *N*-acetylglucosamine, methyl α-*N*-acetyl-D-glucosaminide and the components of bovine cervical mucopolysaccharide and hog gastric mucopolysaccharide are shown at various times of oxidation.

Table 1. *Destruction of components of mucopolysaccharides and related materials by neutral sodium hypochlorite*

Material under investigation (μg./ml.)	Time of oxidation (hr.)	Amount of component in the oxidation mixture (μg./ml.)							
		Hexose by anthrone reaction	Galactose by quantitative paper chromatography	Hexosamine	Reducing sugar	Sialic acid	Amino nitrogen	Fucose	*N*-Acetylhexosamine
Bovine cervical mucopolysaccharide (1000)	0	304	—	245	464	83	67	—	—
	9	298	—	237	450	78	65	—	—
Hog gastric mucopolysaccharide (1000)	0	304	222	287	516	—	50	86	—
	0·5	304	226	290	540	—	48	92	-
	9	283	210	249	450	—	43	76	—
Galactose (113)	0	113	—	—	—	—	—	—	—
	3	109	—	—	—	—	—	—	—
	9	103	—	—	—	—	—	—	—
N-Acetylglucosamine (164)	0								164
	1								155
	18								140
Methyl α-*N*-acetyl-D-glucosaminide (186)	0			104					
	0·5			101					
	43			100					

As determined by the anthrone reaction, oxidation of galactose for 9hr. at 0–4° causes only about 10% destruction of galactose. Deionization of a solution of galactose oxidized for 9hr. followed by paper chromatography in solvent (1) showed the presence of a strong galactose spot with only a faint trail of material behind the main spot. No other major component could be detected by the aniline hydrogen phthalate spray. Oxidation of *N*-acetylglucosamine with hypochlorite for 18hr. at 0° causes the destruction of about 15% of the material as determined by the *N*-acetylhexosamine reaction. Methyl α-*N*-acetyl-D-glucosaminide is even more resistant to neutral hypochlorite, less than 5% of hexosamine being destroyed in up to 43hr. oxidation. Similarly, oxidation of methyl α-D-glucopyranoside, starch and dextran 500 for 9hr. followed by estimation of the glucose content after hydrolysis with glucose oxidase showed no detectable destruction. When glucose was treated similarly and the amount remaining after 9hr. of oxidation measured with glucose oxidase it was found that only 5% of the glucose had been destroyed. The destruction of components of hog gastric mucopolysaccharide and bovine cervical mucopolysaccharide also shown in Table 1 indicate that there is a general non-specific loss of small amounts of all the carbohydrate material. In hog gastric mucopolysaccharide there appears to be a greater destruction of hexosamine than galactose but in bovine cervical mucopolysaccharide the reverse occurs. However, it is doubtful whether this result is significant since these values are dependent on the difference in two analytical values and as such the small destruction observed is probably subject to an appreciable error. That the difference in anthrone values did give a true indication of the amount of galactose destroyed and that oxidation products were not causing any serious error was shown by quantitative paper chromatography. In the cervical mucopolysaccharide very little destruction of sialic acid had occurred, which was rather surprising in view of the labile nature of this component. The amino nitrogen content was lowered slightly after hypochlorite oxidation, but with bovine cervical mucopolysaccharide this decrease was only marginally greater than the experimental error. Hog gastric mucopolysaccharide after a 9hr. hypochlorite oxidation did, however, show a significant decrease in amino nitrogen content. Comparison of amino acid analyses of the oxidized materials with those of the undegraded substances by using the resin-column technique of Moore & Stein (1951, 1954) showed that only arginine was completely lost during the oxidation; further, this amino acid was completely destroyed after only 0·5hr. oxidation. A general non-specific destruction of the amino acids must therefore have occurred since the relative amounts of the other amino acids was unchanged even after oxidation for 9hr.

Oxidation of bovine submaxillary-gland mucoprotein and ovalbumin with hypochlorite followed by reduction of the oxidation products with sodium borohydride did not destroy any appreciable amount of hexosamine. This indicated that the linkage involving the hexosamine in these compounds was stable to hypochlorite.

Depolymerization of macromolecules by hypochlorite. Gel filtration on granulated agar columns was used to follow the depolymerization of a number of proteins, polysaccharides and mucopolysaccharides. To estimate the average molecular weight of the oxidized polysaccharides and mucopolysaccharides the agar columns were first calibrated with dextrans of known molecular-weight ranges. In this way it was found that a 12% (w/w) agar column completely excluded dextran molecules with mol.wt. greater than 40000 but not those with mol.wt. smaller than 10000. A 7% (w/w) column excluded molecules with mol.wt. greater than 250000 but not those with mol.wt. 40000, whereas on a 4% (w/w) column even molecules with mol.wt. as large as 500000 were not excluded and molecules with mol.wt. as large as 10000 were completely included. As an illustration of the usefulness of these columns for approximate molecular-weight determination Fig. 5 shows a plot of peak volume against log(mol.wt.) for a series of dextrans on a 4% (w/w) agar column. When the depolymerization of proteins was studied the columns were calibrated with a set of proteins of known molecular weight, namely bovine serum albumin, haemoglobulin, α-lactalbumin; sucrose was put in to estimate the complete inclusion volume. Large errors could

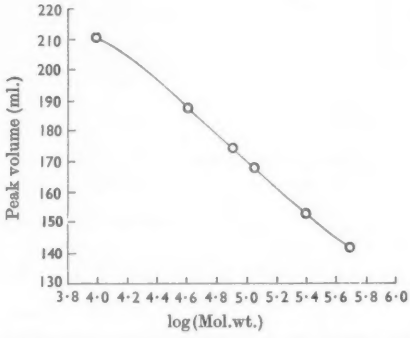


Fig. 5. Plot of peak volume (ml.) against log(mol.wt.) for dextrans on a 4% (w/w) agar column (60cm. × 2·4cm.) with 0·02M-LiCl for elution.

arise by attempting to estimate the depolymerization of proteins on a column calibrated with dextrans because of the difference in shape of the molecules, the proteins being more compact than the rather extended structures of the polysaccharides.

The progressive depolymerization of bovine cervical mucopolysaccharide by oxidation with neutral hypochlorite is shown by elution diagrams on a 7% (w/w) agar column in Fig. 6. Although the molecular weight of the starting material was about 4×10^6 (Gibbons & Glover, 1959), after oxidation for 9hr. a large amount of the material had mol.wt. less than 40000. Even after only 0·5hr. of oxidation the average molecular weight had dropped appreciably. There is obviously a very rapid and extensive depolymerization of the molecule.

The depolymerization of dextran 500, chondroitin sulphate, ovalbumin and serum albumin after oxidation for 9hr. is shown in Fig. 7. Although dextran 500 and chondroitin sulphate have been

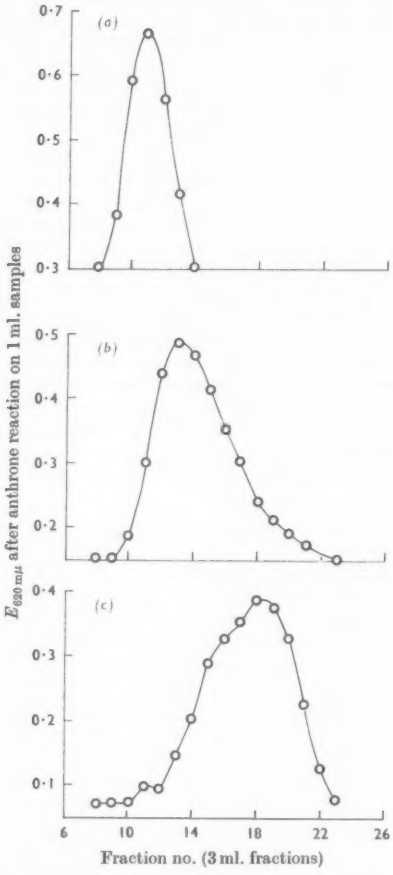


Fig. 6. Gel filtration of bovine cervical mucopolysaccharide on a 7% (w/w) agar column. (*a*) Unoxidized material; (*b*) after oxidation with NaClO at pH 7 and 0–4° for 0·5hr.; (*c*) after oxidation for 9hr.

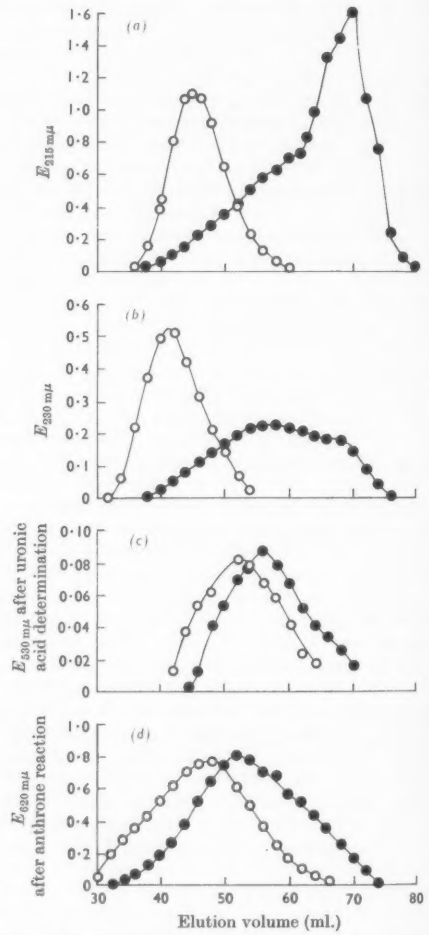


Fig. 7. Gel filtration of ovalbumin, bovine serum albumin, chondroitin sulphate and dextran 500 before (○) and after (●) oxidation with neutral NaClO at 0–4° for 9hr. (*a*) Ovalbumin on a 12% (w/w) agar column; (*b*) bovine serum albumin on a 12% (w/w) agar column; (*c*) chondroitin sulphate on a 4% (w/w) agar column; (*d*) dextran 500 on a 4% (w/w) agar column.

depolymerized to a small extent, the observed depolymerization is not comparable with the extensive depolymerization of ovalbumin and serum albumin under the same conditions.

Fractionation and analysis of hypochlorite-oxidized fragments. Attempted fractionation of hypochlorite-oxidized epithelial mucopolysaccharides by adsorption chromatography on charcoal and ion-exchange chromatography on DEAE-cellulose was unsuccessful, a large amount of the material becoming irreversibly adsorbed on the column media. However, fractionation according to size of the fragments on gel-filtration columns was possible, almost quantitative recoveries being obtained off agar columns. An elution diagram on 12% (w/w) agar of hog gastric mucopolysaccharide (455mg.) oxidized for 9hr. is shown in Fig. 8. The eluates were assayed by extinction at 215mμ and the fractions combined as shown in Table 2. The first two combined fractions were freeze-dried, deionized by extracting the lithium chloride with ethanol and analysed. The third combined fraction, which contained large amounts of salt other than lithium chloride, was made up to a known volume in a standard flask and analyses were carried out on this. In this way the total amount of components in the fraction could be obtained. The analyses of these fractions are shown in Table 2. There is little difference in the ratios of the components in each fraction. The third fraction shows a higher anthrone/hexosamine ratio than the first two but this is probably due to the chloride present in this fraction interfering slightly with the anthrone reaction, causing it to give high results. There is no significant difference in the hexosamine/amino nitrogen ratio in the three fractions, indicating that separation of a protein-rich or carbohydrate-rich fragment has not occurred in this fractionation. The remainder of the third fraction was freeze-dried and fractionated on a Sephadex G-25 column with water for elution; the elution diagram obtained is shown in Fig. 9. Over half of the material was excluded from the Sephadex column, indicating that it was greater than 3000–4000 in mol.wt. Analyses of the fractions

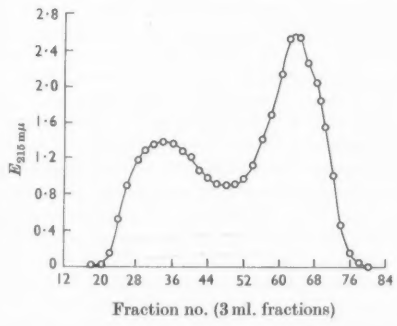


Fig. 8. Fractionation of hog gastric mucopolysaccharide (455mg.) oxidized with neutral NaClO at 0–4° for 9hr. on a 12% (w/w) agar column (60cm. × 2·4cm.) with 0·02M-LiCl for elution.

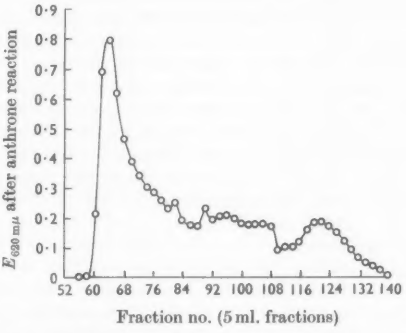


Fig. 9. Fractionation of pooled fractions 54–77 off a 12% (w/w) agar column (see Fig. 8) of hog gastric mucopolysaccharide oxidized with neutral NaClO on a Sephadex G-25 column (110cm. × 3cm.) with water for elution.

Table 2. *Analyses of fractions obtained by fractionating sodium hypochlorite-oxidized hog gastric mucopolysaccharide* (455mg.) *on a* 12% (*w/w*) *agar column* (60cm. × 2·4cm.) (*see Fig.* 8)

The values in parentheses are molar ratios, hexosamine being taken as unity.

	Amount of component (mg.)				
Fractions	Hexose by anthrone reaction	Hexosamine	Reducing sugar	Fucose	Amino nitrogen
20–44	48·5 (0·82)	59·7 (1)	91·6 (1·50)	14·7 (0·27)	9·16 (2·0)
45–53	16·9 (0·98)	17·1 (1)	29·6 (1·67)	4·94 (0·31)	3·79 (2·8)
54–77	51·8 (1·45)	35·6 (1)	70·9 (1·95)	9·68 (0·29)	7·22 (2·6)

Table 3. *Analyses of fractions of sodium hypochlorite-oxidized hog gastric mucopolysaccharide off a Sephadex G-25 column (see Fig. 9)*

The values in parentheses are molar ratios, hexosamine being taken as unity.

Fractions	Amount of component (mg.)				
	Hexose by anthrone reaction	Hexosamine	Reducing sugar	Fucose	Amino nitrogen
58–73	17·2 (0·96)	18·3 (1)	33·1 (1·8)	4·48 (0·27)	2·61 (1·9)
74–88	7·61 (0·98)	7·66 (1)	14·7 (1·9)	1·97 (0·28)	1·13 (1·9)
89–100	3·71 (1·1)	3·41 (1)	6·54 (1·9)	1·10 (0·35)	0·81 (3·1)
101–110	1·92 (1·2)	1·62 (1)	3·22 (2·0)	0·62 (0·42)	0·63 (5·0)

obtained are shown in Table 3. Here again there is little difference in anthrone/hexosamine ratio in the various fractions, but the amino nitrogen/hexosamine ratio does increase appreciably as the fragments become smaller. However, the total amount of these small fragments is very small, being less than 5% of the starting material. A partial separation of SO_4^{2-} and Cl^- ions occurred on this Sephadex column. There is some ionic material with a high u.v.-absorption travelling slower than the Cl^- ion, but this did not contain any sugar or nitrogenous material and was not examined any further.

Fractionation of bovine submaxillary-gland mucoprotein and bovine cervical mucopolysaccharide after hypochlorite oxidation on 12% (w/w) agar columns gave elution patterns similar to that in Fig. 9, but in these cases the second peak was smaller, probably owing to the starting material being a larger molecule.

Similar fractions were combined and analysed as for hog gastric mucopolysaccharide. With bovine cervical mucopolysaccharide there was little difference in the ratios of the components present in the first two fractions, which were similar to the starting material. In the third fraction the high salt concentration relative to the material being analysed made reliable analyses difficult and an apparent increase in anthrone and amino nitrogen was obtained. This was probably due to interference by salt, since the same fraction did not show such an increase when the products were isolated by a procedure designed to give very little salt, namely method (*a*) in the Methods section. When the products of oxidation of bovine submaxillary-gland mucoprotein were isolated by method (*a*) and fractionated on a 12% (w/w) agar column the hexosamine/amino nitrogen ratios did not vary very much in the three fractions, indicating that the hypochlorite had not attacked the linkage joining the disaccharide prosthetic groups to the main polypeptide chain, nor had it liberated appreciable amounts of an oligosaccharide-rich fragment.

DISCUSSION

It is rather surprising that sugars with free reducing groups are relatively stable to hypochlorite under the conditions used in this study, since one of the accepted methods of oxidizing a free reducing group to the acid is by means of hypohalites. However, these oxidations are usually carried out at a more extreme pH value, at a higher temperature and with hypobromite or hypoiodite. The amount of ClO^- ion, dissolved chlorine and undissociated hypochlorous acid in hypochlorite solutions depends very markedly on the pH (Green, 1948), and these three oxidizing agents react differently. The stability of the glucosaminide linkage to neutral hypochlorite is evident from the lack of chlorine uptake by methyl α-*N*-acetyl-D-glucosaminide and the negligible destruction of glucosamine linked in this way. Free glucosamine, however, is rapidly oxidized, as might be expected, the attack presumably being at the free amino group, since *N*-acetylglucosamine is relatively stable. Whistler & Schweiger (1957) investigated the oxidation of amylopectin with hypochlorite at various pH values and found that attack was most rapid at pH7. The main products of oxidation were glyoxylic acid and erythronic acid, indicating that cleavage of the carbon–carbon bond had occurred between C-2 and C-3. However, these experiments were carried out at 25° for long periods of time, and examination of the oxidation of glucose, methyl α-D-glucopyranoside, dextran and starch with neutral hypochlorite at 0–4° showed that very little destruction of glucose had occurred in 9hr.

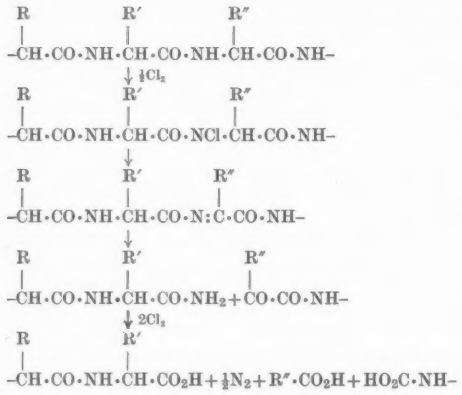
An examination of the hypochlorite uptake by amino acids and peptides has shown that, whereas a rapid uptake is observed in the presence of a free amino group, attack of a peptide bond is also possible. This is illustrated by the hypochlorite reduction by dioxopiperazine and *N*-benzoylglycine methyl ester. Although papers have appeared on the oxidation of amino acids at various pH values,

little is known about the effect of neutral hypochlorite on the peptide bond. It is known that, under acidic conditions, amino acids and peptides give *N*-chloro derivatives, which are then further oxidized (Wright, 1936), and a number of authors have described the reaction of hypohalite with proteins, peptides and amino acids (Goldschmidt, Wiberg, Nagel & Martin, 1927; Herken & Schunk, 1949; Stankovic & Vasátko, 1960; McGregor & Carpenter, 1962). McGregor & Carpenter (1962) showed that at a weakly alkaline pH (9·4) the free amino group of peptides is oxidatively deaminated to give *N*-(α-oxoacyl)-peptides, and that these are very readily cleaved on further oxidation with hydrogen peroxide.

Although a small non-specific destruction of the sugar components of epithelial mucopolysaccharides occurred this would be insufficient to explain the large uptake of hypochlorite. There was a small decrease in nitrogen released as amino nitrogen on hydrolysis and an amino acid analysis revealed that arginine had been completely destroyed. Arginine forms rather a small proportion of the total amino acids in the molecule, but this does rule out the possibility of an oligosaccharide side chain being attached to the guanidyl residue of arginine. Such a linkage has been suggested by Micheel & Hulsmann (1960). The rapid disappearance of arginine is most probably due to the oxidation of the guanidine group and this need not imply scission of any peptide bond.

The hexosamine content of bovine submaxillary-gland mucoprotein is not substantially decreased by oxidation with neutral hypochlorite even after reducing the oxidation products with sodium borohydride. This indicates that sugars linked glycosidically to serine and threonine are not preferentially attacked by hypochlorite since most of the galactosamine in bovine submaxillary-gland mucoprotein has been shown to be linked in this way (Hashimoto, Tsuiki, Nisizawa & Pigman, 1963; Anderson *et al.* 1964; Tanaka, Bertolini & Pigman, 1964; Tanaka & Pigman, 1965). The destruction of serine and threonine in bovine cervical mucopolysaccharide under mild alkaline conditions (R. A. Gibbons & G. P. Roberts, unpublished work) suggests that similar linkages are present but here again little decrease in the hexosamine content was observed, and no sugar component was preferentially destroyed. The *N*-acylglycosylamine linkage present in ovalbumin (Johansen, Marshall & Neuberger, 1961; Nuenke & Cunningham, 1961) also appears to be resistant to attack by neutral hypochlorite since no liberation of oligosaccharide units from ovalbumin was observed. Further evidence of the stability of this linkage is provided by the lack of destruction of any appreciable amount of the hexosamine residues on reducing the oxidized products with sodium borohydride. The loss of viscous properties of cervical mucus during hypochlorite oxidation observed by Boyland (1946) may be attributed to the depolymerization of the mucopolysaccharide component present in the mucus. A very rapid and extensive depolymerization of this mucopolysaccharide occurs, the mol.wt. being decreased from 4×10^6 to below 250000 in 30min., whereas after 9hr. the average mol.wt. is well below 40000 and a large amount of the material has mol.wt. less than 10000. Although polysaccharides and connective-tissue mucopolysaccharides such as dextran and chondroitin sulphate are depolymerized to a small extent by hypochlorite, it is clear that glycosidic, glycosiduronic and *N*-acetylhexosaminidic linkages are attacked at a considerably lower rate than peptide bonds; in fact, the elution diagrams from suitably calibrated agar columns indicate that ovalbumin and serum albumin fragment approximately 5 and 6 times as rapidly respectively as dextran. Our gel-filtration data for chondroitin sulphate and cervical mucopolysaccharide do not allow deduction of the molecular weights of the depolymerized products of these samples since our only reference substances for these two materials were fractionated dextran samples. Nevertheless, it is justifiable to state that chondroitin sulphate breaks up slowly and cervical mucopolysaccharide quite rapidly in this respect the latter resembles protein rather more than polysaccharide. From this it can be inferred that the peptide part of the mucopolysaccharide is concerned with the integrity of the molecule, possibly as a long peptide chain with oligosaccharide prosthetic groups.

The relative unreactivity of methyl α-*N*-acetyl-D-glucosaminide and *N*-acetyl- or *N*-glycollylneuraminic acid residues indicates that, although they readily form *N*-chloro derivatives, *N*-substituted amides in general do not react further with hypochlorite under the conditions we have used. It is suggested that, in an *N*-chlorinated protein, the presence of a peptide carbonyl in a β-position to the chloroamide nitrogen atom facilitates the elimination of hydrogen chloride with the formation of a labile acylimino group. This then splits with the formation of an α-oxoacyl structure and an amide, and the latter is then rapidly oxidized to nitrogen and a carboxyl residue. The former may also split to give a carboxylic acid and a formyl or carboamino polypeptide. In either case, the amino end of the resultant split protein is protected against further rapid oxidation. This reaction scheme shown in Scheme 1 is an extension of that of McGregor & Carpenter (1962). The resistance of *N*-aspartoamidoglycoside to oxidation is explained by this reaction scheme. It was realized that, if this reaction scheme were correct, poly-L-proline



Scheme 1. Possible reaction scheme for the oxidation of the peptide linkage by neutral hypochlorite.

should be unattacked by neutral hypochlorite. When this was tested with synthetic poly-L-proline the hypochlorite uptake was found to be very much less than that of other peptides and proteins, thus providing further evidence in favour of this reaction sequence.

The destruction of one amino acid residue is probably therefore accompanied by the splitting of the peptide bond and the reduction of 2 or 2·5 mol. of chlorine/mol., whereas it seems likely that 0·5–1 mol./mol. would suffice for a polysaccharide (Whistler & Schweiger, 1957). This difference, together with the rapid oxidation of free amide and guanidine groups that occur in proteins, explains why the vast difference in hypochlorite uptake between proteins and polysaccharides is accompanied by a rather moderate difference in the rates of destruction of the component residues (Table 1).

The ready fragmentation of epithelial mucopolysaccharides by hypochlorite is consistent with a structure composed of a long peptide chain with oligosaccharide side chains. On hypochlorite oxidation the peptide chain would be attacked, producing small fragments that would be analytically similar to the intact mucopolysaccharide. The objection to the presence of a long polypeptide chain raised by Gibbons & Roberts (1963) has now been removed (G. P. Roberts, unpublished work), since it has been shown that proteins containing substituted hydroxyamino acids are extremely labile to alkali and are rapidly depolymerized by alkali. The presence of serine and threonine glycosides in epithelial mucopolysaccharides has now been established by several workers (Hashimoto *et al.* 1963; Anderson *et al.* 1964; Bhavanandan, Buddecke, Carubelli & Gottschalk, 1964; Harbon, Herman, Rossignol, Jollès & Clauser, 1964; Tanaka *et al.* 1964).

A structure composed of a long peptide chain with oligosaccharide prosthetic groups is not the only structure that would explain the observations in this paper. If it is accepted that peptide is concerned with the integrity of the molecule then a structure composed of alternating chains of peptide and sugars would also explain the experimental results presented above. Although short oligosaccharide prosthetic groups are considered possible, short polysaccharide chains alternating between short peptide chains are considered less likely because of the extremely large number of sugar–peptide links that would be required. In view of the stability of the glucosidic and glucosaminidic linkage to neutral hypochlorite, if a long polysaccharide chain existed in the molecule it would be relatively resistant to oxidation and it would be expected that such a chain could be isolated on prolonged oxidation. However, there was no increase in the polysaccharide content of the largest fragments isolated by gel filtration after prolonged oxidation, thus indicating that no long polysaccharide chain existed in the molecule. There is, however, a possibility that a small number of the relatively short oligosaccharide prosthetic groups may be linked to more than one peptide chain, thus giving a large network, but there is no evidence for or against this. In view of these possibilities and uncertainties, although a continuous polypeptide chain with numerous *O*-glycosidically linked oligosaccharide side chains seems likely, further investigation is required before an overall structure can be ascribed to the epithelial mucopolysaccharides with any degree of certainty.

This work was supported by a grant from the Population Council, Rockefeller Institute, New York, to whom we express our grateful thanks.

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Effect of Sex and Gonadal Hormones on Rat Plasma Lipids During the Development of an Essential Fatty Acid Deficiency

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(*Received* 2 *August* 1965)

1. Male, female and castrated rats treated with oestradiol (30μg./week) or testosterone (2mg./week) were given an essential fatty acid-deficient diet containing 10% of hydrogenated coconut oil for 9 weeks. The concentrations and fatty acid composition of plasma phospholipids, cholesteryl esters and triglycerides were determined. 2. Between the second and third weeks of the deficiency, concentrations of plasma cholesteryl esters, phospholipids and triglycerides decreased, then remained relatively constant. There were no significant differences between males and females, but oestradiol caused a significant rise in plasma phospholipids and triglycerides as compared with testosterone-treated animals. 3. During the first 2 weeks of the deficiency, linoleic acid in the plasma lipids of all groups decreased to low concentrations and changed very little thereafter. 4. Female rats maintained higher percentages and concentrations of arachidonic acid and stearic acid in plasma phospholipids and arachidonic acid in cholesteryl esters than did males. Males had higher proportions of eicosatrienoic acid and oleic acid. There was no sex difference in the fatty acid composition of plasma triglycerides. 5. Oestradiol-treated rats had concentrations of cholesteryl and phospholipid arachidonate comparable with those of female rats and higher than the testosterone-treated group. Eicosatrienoic acid in the oestradiol–treated rats was high and resembled that of the male rats, apparently because of the higher concentration of plasma phospho lipids in this group. 6. Supplementation of the essential fatty acid-deficient rats with linoleate restored plasma cholesteryl and phospholipid linoleate and arachidonate nearly to normal concentrations in a single day. The increase in arachidonic acid in these fractions was accompanied by a similar quantitative decrease in eicosatrienoic acid. 7. These sex differences appear to be related to the smaller size of the female rat and to a more direct influence of oestradiol on the formation or maintenance of phospholipids rich in arachidonic acid.

There is ample evidence that sex hormones influence lipid metabolism in mammals (Gyorgy, Rose & Shipley, 1947; Fillios, Kaplan, Martin & Stare, 1958; Okey & Lyman, 1956; Olson & Vester, 1960) and the fatty acids of tissue and blood lipids (Coleman, Chen & Alfin-Slater, 1958; Morton & Horner, 1961; Horner & Morton, 1961; Aftergood & Alfin-Slater, 1965; Bowers & Schelly, 1965). Okey, Ostwald, Shannon & Tinoco (1962) reported that female rats on diets containing cholesterol had higher proportions of arachidonic acid and stearic acid in their plasma phospholipids than similarly treated male rats; the difference occurred principally in the lecithins (Lyman, Shannon, Ostwald & Miljanich, 1964*b*). Monsen, Okey & Lyman (1962) showed that neither cholesterol nor diets varying widely in linoleic acid influenced this sex difference in plasma phospholipid fatty acid composition. Oestradiol injected into castrated male rats produced a comparable increase in the stearic acid and arachidonic acid of the lecithins. These results suggested that the female rat may be able to mobilize or to utilize linoleic acid in the conversion of arachidonic acid better than the male. Consequently, an experiment was conducted with male and female rats, in which carcass linoleate and liver-lipid fatty acids were analysed during the development of an essential fatty acid deficiency (Ostwald, Bouchard, Miljanich & Lyman, 1965). The results failed to show any sex differences in the total amount of linoleic acid mobilized or total amount of arachidonic acid in liver-lipid fractions, but female rats maintained somewhat higher concentrations of linoleate in their adipose tissue and

higher proportions of arachidonic acid and stearic acid in their liver phospholipids than males. The effects of sex and the gonadal hormones, oestradiol and testosterone, on linoleate and arachidonate in plasma phospholipids, cholesteryl esters and triglycerides during the development of EFA* deficiency were investigated on the same animals.

MATERIALS AND METHODS

Details of the diet, treatment of animals and design of the experiment were presented by Ostwald *et al.* (1965). Intact female and male rats and castrated male rats, injected with the hormone vehicle (hydrogenated coconut oil), oestradiol (30 μg./week) and testosterone (2mg./week), were given for 64 days a diet free of essential fatty acids but containing 10% of hydrogenated coconut oil. Some of the intact male and female animals were kept on this EFA-deficient diet for a total of about 17 weeks (119 days), after which groups of rats of both sexes were given the basic diet but with the 10% of hydrogenated coconut oil replaced by an equivalent amount of safflower oil.

Plasma-lipid extraction, fractionation and analyses. The blood was centrifuged (at 5°) immediately, and the plasma obtained was extracted by adding it dropwise to 10 vol. of 95% ethanol containing 1mg. of quinol. Plasma from each animal (3·5–5·0ml.) was treated individually except those in the zero-time group, where pools of plasma (5·5ml.) from males or females were analysed in duplicate. The ethanol extract was then filtered and the residue was re-extracted twice with 10 vol. of 95% ethanol–diethyl ether (3:1, v/v). The combined filtrates were concentrated on a rotary vacuum evaporator below 50° to about 1 ml. The aqueous residue was extracted three times with light petroleum (b.p. 30–60°) at room temperature, and stored at 0° until analysed. The residue was extracted further with three rinses of warm chloroform and three rinses of cold chloroform (Stevan & Lyman, 1963). The combined chloroform extracts were also stored at 0° until analysed.

The light-petroleum extracts were centrifuged, and the residue was washed with chloroform. The light-petroleum and chloroform extracts were then combined and evaporated to dryness. The lipids from a single plasma sample were dissolved in 10ml. of light petroleum. Total cholesterol (Sperry & Webb, 1950) and phosphorus (Sumner, 1944) were determined on 2ml. of this solution. The remainder of the extract was then fractionated into cholesteryl esters, triglycerides, free cholesterol and phospholipids by the method of Lis, Tinoco & Okey (1961). Recoveries of cholesterol and phospholipid were never less than 90% and mostly were higher.

A known quantity of heptadecanoic acid was added to the lipid fractions as an internal standard, so that the amount of each fatty acid in a fraction could be determined (Tinoco, Shannon, Miljanich, Lyman & Okey, 1962). Where independent measurements of cholesterol or phosphorus could be made, such as in the esterified cholesterol or the phospholipid fractions, the internal standard was used mainly to check the total recovery of the fatty acids. Plasma triglycerides, however, were determined completely by means of the internal standard. Fatty acids from the lipid fractions were methylated and then determined by gas–liquid chromatography (Lyman *et al.* 1964*b*). Plasma concentrations for the phospholipid fatty acids were calculated on the basis of the analyses of phosphorus in the phospholipid fraction × 17·3 × weight (%) of the fatty acid determined by gas–liquid chromatography. Esterified cholesterol fatty acid concentrations were calculated from cholesterol determined in the cholesteryl ester fractions × 0·73 × weight (%) of fatty acids in the fraction. Carbon-chain lengths and number of double bonds in the eicosatrienoic acids were established by hydrogenation and by comparison with known fatty acids.

Statistical comparisons of the results were made by the *t* test (Snedecor, 1956).

RESULTS

Concentrations of plasma cholesterol, phospholipid and triglyceride. Fig. 1 shows the concentrations of plasma cholesterol, phospholipid and triglyceride in the experimental groups during the development of EFA deficiency. Apart from the slight fall between 16 and 21 days in the free and esterified cholesterol of intact females and males, the EFA deficiency had very little effect on the concentration of either free or esterified cholesterol in any of the groups. Although male and testosterone-treated rats appeared to have consistently less free cholesterol than did females and castrated rats injected with oestradiol, the differences were not significant owing to wide variations in individual values. In intact rats there was an initial increase and then a fall in phospholipid concentration in both sexes between the second and third weeks of the EFA deficiency. Phospholipid concentrations in males appeared to decrease slightly during the deficiency, whereas in females phospholipids remained about the same from the fourth week of the deficiency to the termination of the experiment at 9 weeks. However, after 17 weeks, both sexes had greatly elevated amounts of phospholipids, which decreased when linoleic acid was fed (Fig. 1*c*). The changes in the phospholipid concentrations in testosterone-treated rats were similar to those of the male animals. However, oestradiol caused a sharp increase in the plasma phospholipid, which was maintained significantly above the concentrations of the testosterone-treated group and those of the intact animals.

Plasma triglycerides in female rats declined in the first 3 weeks of the EFA deficiency, then remained at about 20–30mg./100ml. of plasma over the 9-week interval. Males showed only a slight decrease in this lipid during the onset of the EFA deficiency. Again, as with the phospholipid, oestradiol treatment increased plasma triglycerides significantly above the value in the intact females and in the group injected with testosterone. The concentrations in the latter group were similar to

* Abbreviation: EFA, essential fatty acids.

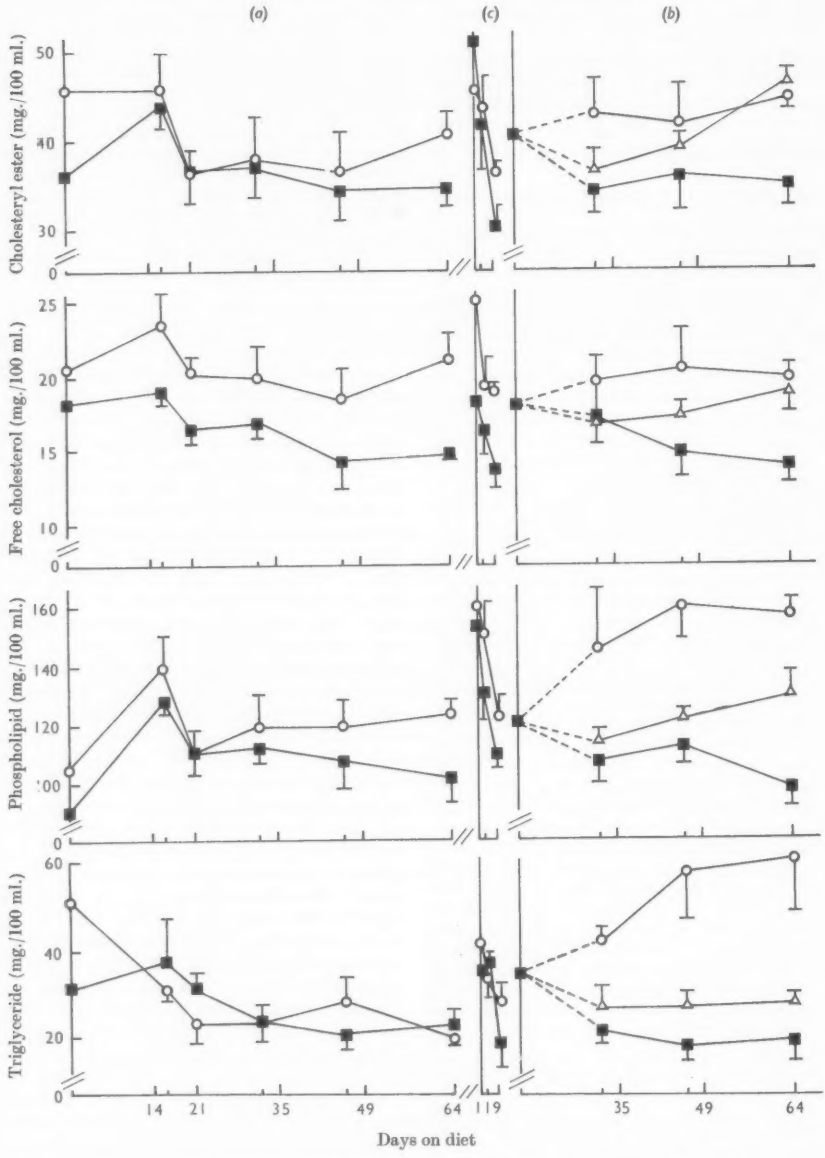

Fig. 1. Plasma cholesterol, phospholipids and triglycerides in rats during development of EFA deficiency. (*a*) Intact males (■), females (○). (*b*) Castrated males treated with testosterone (■), oestradiol (○) or the hormone vehicle (△). Castrations were performed and hormone injections begun approximately 2 weeks after animals were placed on the EFA-deficient diet. (*c*) Results of re-feeding linoleate to male and female rats after 17 weeks on the EFA-deficient diet. Intervals are for 1 and 3 days after feeding with a diet containing safflower oil. Vertical lines on Figures are half standard error of the mean from five or more rats/group/time period.

those of the intact males. Castrated rats injected only with the oil used to dissolve the hormone maintained concentrations of cholesteryl esters, phospholipids and triglycerides that were generally between the values of the oestradiol- and testosterone-treated rats. Therefore plasma lipids of this group usually were not significantly different from those of the other two groups.

After 17 weeks, all plasma-lipid fractions in rats on the deficient diet had increased above the 9-week values in both sexes (Fig. 1*c*). Feeding with linoleic acid caused an abrupt decrease in those concentrations, so that after 3 days they were as low as or lower than at the beginning of the experiment. In EFA deficiency therefore linoleic acid significantly decreased not only plasma cholesterol but also plasma phospholipids and triglycerides.

Phospholipid fatty acids. Table 1 shows the percentages of the unsaturated fatty acids in the plasma phospholipids. Initially, linoleic acid and arachidonic acid were the principal unsaturated fatty acids in this fraction. By the ninth week of the deficiency, however, the percentages of linoleic acid and, to a less extent, arachidonic acid, had decreased, whereas oleic acid and eicosatrienoic acid had greatly increased. Males generally had

Table 1. *Effect of EFA deficiency in male, female and hormone-treated rats on the unsaturated fatty acids of plasma phospholipids*

Fatty acids are expressed as percentages of total methyl esters determined. Longer-chain fatty acids represented about 4% of the total fatty acids. Means ± S.E.M. are from five or more rats/group/time period.

	Fatty acids (%, by wt.)				
			Castrated rats		
Days on diet	Females	Males	Oil	Oestradiol	Testosterone
			16:1		
0	1·4	2·4	—	—	—
16	1·7±0·2	2·0±0·2	—	—	—
21	1·7±0·2	2·6±0·9	—	—	—
32	2·1±0·2	2·8±0·4	2·2±0·2	1·5±0·3	2·2±0·08
46	2·1±0·2	2·7±0·2	2·5±0·1	2·0±0·5	2·4±0·3
64	1·9±0·9	2·6±0·1	2·3±0·3	1·7±0·3	2·1±0·1
			18:1		
0	7·5	7·5	—	—	—
16	11·8±1·1	12·6±0·7	—	—	—
21	12·8±0·9	16·3±0·8	—	—	—
32	15·0⊥0·7	15·8±0·6	15·3±0·5	13·5±0·4	15·2±0·8
46	14·8±1·8	18·3±0·6	16·5±0·6	15·4±0·7	17·1±0·7
64	15·6±0·4	19·3±0·6	18·2±0·8	13·7±0·6	16·0±1·6
			18:2		
0	13·2	12·5	—	—	—
16	6·7±0·4	7·6±0·1	—	—	—
21	6·4±0·4	7·3±0·8	—	—	—
32	5·6±0·7	5·3±1·0	7·0±0·7	6·4±0·8	8·7±0·4
46	5·1±0·2	3·8±0·5	4·6±0·4	4·4±0·7	6·2±1·0
64	4·8±0·3	4·6±0·8	4·0±0·5	4·5±0·1	5·3±0·4
			20:4		
0	18·9	16·4	—	—	—
16	26·8±2·6	23·4±1·0	—	—	—
21	28·9±3·0	13·6±1·2	—	—	—
32	22·6±2·3	14·1±1·2	19·3±1·4	19·2±2·9	16·1±2·1
46	21·4±3·4	8·2±0·8	13·1±1·2	15·0±2·0	13·6±1·4
64	18·5±2·0	10·6±1·9	12·3±1·7	18·6±0·7	10·6±1·0
			20:3		
0	—	—	—	—	—
16	5·4±1·6	6·1±0·9	—	—	—
21	5·1±1·4	12·6±0·9	—	—	—
32	12·5±3·2	19·8±2·4	14·0±2·2	14·3±3·4	12·3±1·1
46	12·7±2·0	24·9±2·0	20·1±1·8	19·7±1·9	18·9±3·1
64	17·3±1·6	21·8±2·1	20·2±1·2	20·1±1·1	22·2±0·8

higher proportions of oleic acid and eicosatrienoic acid than females, whereas females maintained significantly higher proportions of arachidonic acid than males ($P < 0{\cdot}02$ at 21, 32 and 46 days; $P < 0{\cdot}05$ at 64 days). The major decrease in the percentage of linoleic acid in this lipid fraction occurred during the first 2 weeks of the deficiency, with only a slight change thereafter. Palmitoleic acid was a relatively minor component of the phospholipids, and its proportion was unaffected by sex or by EFA deficiency. In general, the proportions of plasma phospholipid fatty acids in the oestradiol- or testosterone-treated animals resembled those in the respective intact controls. However, the differences between percentages of arachidonic acid in the oestrogen- and androgen-treated groups were not so large as those between female and male rats, and values were significant only at 64 days ($P < 0{\cdot}01$). Further, the proportion of eicosatrienoic acid was higher in the oestradiol group than in the females, and was therefore nearly identical with that in the testosterone-injected animals.

Fig. 2 shows the concentrations of linoleic acid, arachidonic acid and eicosatrienoic acid in the plasma phospholipids of the five groups of animals. Throughout the experiment linoleic acid decreased only about 5 mg./100 ml., and to the same value in both sexes. Sex hormones did not influence its ultimate concentration. The concentration of arachidonic acid was significantly higher in females

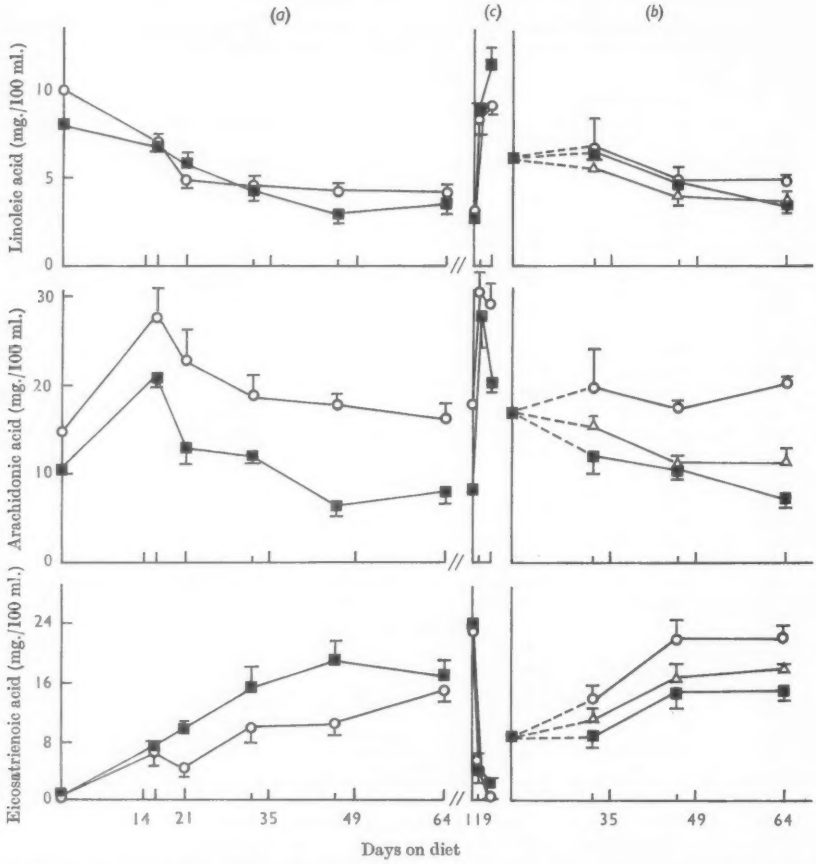

Fig. 2. Concentrations of linoleic acid, arachidonic acid and eicosatrienoic acid in plasma phospholipids of rats during development of EFA deficiency. For explanation of symbols see Fig. 1.

Table 2. *Palmitic acid and stearic acid in plasma phospholipids, esterified cholesterol and triglycerides of EFA-deficient male, female and hormone-treated rats*

Fatty acids are expressed as percentages of total methyl esters determined. Lauric acid and myristic acid represented less than 2% of phospholipid, 3% of esterified cholesterol and 6% of triglyceride fatty acids. Means of palmitic acid or stearic acid from five or more rats/group/time period are shown. Standard errors for palmitic acid and stearic acid in the phospholipids were generally less than 10% of the mean.

					Castrated rats					
	Females		Males		Oil		Oestradiol		Testosterone	
Days on diet	(%, by wt.)	(mg./100 ml.)	(%, by wt.)	(mg./100 ml.)	(%, by wt.)	(mg./100 ml.)	(%, by wt.)	(mg./100 ml.)	(%, by wt.)	(mg./100 ml.)
					Phospholipid					
					16:0					
0	25·5	—	28·5	—	—	—	—	—	—	—
16	19·0	—	22·4	—	—	—	—	—	—	—
21	18·2	—	22·0	—	—	—	—	—	—	—
32	16·2	(13·0)	18·8	(14·8)	17·2	(14·0)	16·0	(16·5)	19·7	(15·4)
46	15·8	(13·4)	17·8	(13·2)	17·9	(15·1)	16·9	(18·8)	18·4	(14·5)
64	15·2	(13·2)	18·0	(13·8)	17·9	(16·2)	14·4	(15·8)	17·6	(12·0)
					18:0					
0	28·9	—	28·9	—	—	—	—	—	—	—
16	25·2	—	21·0	—	—	—	—	—	—	—
21	24·3	—	20·7	—	—	—	—	—	—	—
32	21·3	(17·4)	19·4	(16·0)	21·2	(16·9)	25·5	(25·7)	20·4	(15·4)
46	23·1	(19·4)	18·9	(14·3)	20·2	(17·0)	22·5	(25·1)	19·2	(14·9)
64	22·5	(19·6)	18·9	(14·5)	19·9	(17·9)	22·8	(24·6)	20·8	(14·2)
					Esterified cholesterol					
					16:0					
0	11·3	—	11·4	—	—	—	—	—	—	—
16	8·8	—	10·9	—	—	—	—	—	—	—
21	8·8	—	11·1	—	—	—	—	—	—	—
32	8·0	—	9·2	—	9·0	—	8·6	—	10·9	—
46	9·0	—	9·8	—	8·7	—	8·5	—	10·3	—
64	7·8	—	10·0	—	9·7	—	9·5	—	10·4	—
					18:0					
0	2·9	—	3·4	—	—	—	—	—	—	—
16	2·5	—	3·1	—	—	—	—	—	—	—
21	3·0	—	3·1	—	—	—	—	—	—	—
32	2·4	—	2·4	—	3·0	—	4·3	—	3·3	—
46	3·0	—	3·2	—	3·2	—	5·6	—	3·9	—
64	3·1	—	3·8	—	3·8	—	6·3	—	4·3	—
					Triglyceride					
					16:0					
0	18·9	—	20·5	—	—	—	—	—	—	—
16	28·0	—	28·2	—	—	—	—	—	—	—
21	26·9	—	27·7	—	—	—	—	—	—	—
32	24·8	(5·3)	27·2	(5·1)	26·8	(6·9)	27·0	(10·6)	25·8	(5·3)
46	26·3	(7·2)	26·4	(5·1)	25·5	(6·7)	26·7	(14·7)	25·2	(4·2)
64	24·1	(4·5)	26·4	(5·7)	26·2	(6·9)	27·2	(15·9)	25·4	(4·6)
					18:0					
0	4·9	—	6·4	—	—	—	—	—	—	—
16	5·4	—	5·4	—	—	—	—	—	—	—
21	4·9	—	4·8	—	—	—	—	—	—	—
32	5·2	(1·1)	3·8	(0·7)	4·8	(1·1)	3·5	(1·3)	4·4	(0·9)
46	4·6	(1·2)	5·3	(1·0)	4·6	(1·1)	3·2	(1·5)	4·9	(0·8)
64	5·7	(1·1)	4·9	(1·0)	4·5	(1·1)	3·7	(2·2)	5·1	(0·9)

than in males within 3 weeks after giving the EFA-deficient diet ($P < 0.02$ at 21, 46 and 64 days). Oestradiol-injected rats also maintained plasma phospholipid arachidonate at values comparable with those of the female, whereas oil or testosterone injections kept the values of this fatty acid significantly below those of either the oestradiol-treated or female rats ($P < 0.01$ at 46 days for both groups). Although the concentration of eicosatrienoic acid rose more rapidly and was consistently higher in male rats than in females, the differences between groups were significant only at 21 and 46 days ($P < 0.02$). Since treatment with oestradiol increased the concentration of phospholipid arachidonate, the greater increase of eicosatrienoic acid ($P < 0.02$ at 64 days) in this group as compared with that in the rats injected with testosterone was unexpected. Concentrations of eicosatrienoic acid in this instance more closely resembled those in intact males than in the females.

Giving linoleic acid to these animals (Fig. 2c) resulted in a sudden increase in plasma-phospholipid linoleate and arachidonate in just 1 day. During the same time, eicosatrienoic acid decreased

Table 3. *Effect of EFA deficiency in male, female and hormone-treated rats on the unsaturated fatty acids of plasma esterified cholesterol*

Fatty acids are expressed as percentages of total methyl esters determined. Longer-chain fatty acids represented only about 3% of the total fatty acids. Means ± S.E.M. are from five or more rats/group/time period.

	Fatty acid (%, by wt.)				
			Castrated rats		
Days on diet	Females	Males	Oil	Oestradiol	Testosterone
			16:1		
0	4·8	4·4	—	—	—
16	8·6±0·8	8·6±0·6	—	—	—
21	6·9±0·8	10·4±0·6	—	—	—
32	9·4±0·7	10·9±0·9	9·8±0·4	6·7±0·3	8·8±0·5
46	10·6±0·9	11·4±1·0	9·8±0·5	8·2±0·7	9·2±0·7
64	8·9±0·8	11·2±0·6	10·2±0·7	7·2±0·8	9·0±0·8
			18:1		
0	10·1	10·0	—	—	—
16	11·6±0·7	16·1±1·3	—	—	—
21	11·4±1·1	20·1±1·5	—	—	—
32	13·4±0·4	17·2±0·5	15·8±0·9	12·8±0·4	18·3±1·8
46	14·0±0·7	20·9±0·8	16·7±0·6	15·7±1·3	19·3±1·0
64	13·8±1·0	19·7±0·8	18·0±0·7	14·2±0·6	20·0±1·7
			18:2		
0	15·6	15·4	—	—	—
16	4·9±0·3	6·3±0·5	—	—	—
21	5·6±1·2	6·7±0·2	—	—	—
32	5·7±0·3	5·7±0·8	6·4±0·5	6·1±0·4	7·8±0·4
46	4·9±0·4	4·3±0·4	4·8±0·5	4·6±0·6	5·8±0·6
64	4·5±0·5	5·1±0·2	4·4±0·1	4·6±0·2	5·1±0·5
			20:4		
0	40·4	46·5	—	—	—
16	49·7±4·1	42·7±1·8	—	—	—
21	49·6±4·3	26·8±2·3	—	—	—
32	41·9±4·5	26·4±3·8	36·0±3·2	39·2±3·9	31·6±3·9
46	37·2±2·0	14·7±1·6	27·1±2·1	29·4±4·0	23·8±3·1
64	35·9±3·9	19·0±1·1	23·7±2·7	32·2±3·2	18·9±2·7
			20:3		
0	—	—	—	—	—
16	6·8±1·7	6·5±1·0	—	—	—
21	6·8±1·4	15·8±1·6	—	—	—
32	12·5±2·8	20·7±2·3	13·4±2·3	16·6±4·3	12·1±1·2
46	13·7±2·6	26·8±1·8	21·9±1·7	21·4±2·8	18·9±2·7
64	17·5±1·6	22·0±2·1	22·4±1·6	20·4±1·3	23·9±0·6

to less than 5mg./100ml. The rates at which these changes occurred apparently were not influenced consistently by sex of the animals.

Palmitic acid and stearic acid were the major saturated fatty acids present in the plasma phospholipids (Table 2). The proportions of both decreased rapidly during the first 2 weeks of the deficiency, with a much slower decline thereafter. The only sex difference in these fatty acids was a higher proportion of stearic acid in females than in males ($P < 0{\cdot}01$ at 16, 21, 46 and 64 days). Oestradiol apparently produced a similar but less pronounced effect on the proportion of phospholipid stearic acid. After the first 3 weeks of the EFA-deficient diet, the concentration (values in parentheses, Table 2) of both fatty acids remained essentially constant in all groups. Although palmitic acid concentrations were similar for both sexes, stearic acid concentrations in females and oestradiol-treated rats were significantly higher than in males and testosterone-injected rats (females versus males, $P < 0{\cdot}05$ at 16, 46 and 64 days; oestradiol versus testosterone, $P < 0{\cdot}05$ at 32, 46 and 64 days). In this respect therefore stearic acid behaved in a manner similar to that of arachidonic acid (Fig. 2).

Cholesteryl ester fatty acids. The percentages of the unsaturated fatty acids in plasma cholesteryl esters are shown in Table 3. The esterified cholesterol fraction was only about one-third the size of the phospholipid fraction and had higher proportions of palmitoleic acid and arachidonic acid. The changes in the proportions of oleic acid, linoleic

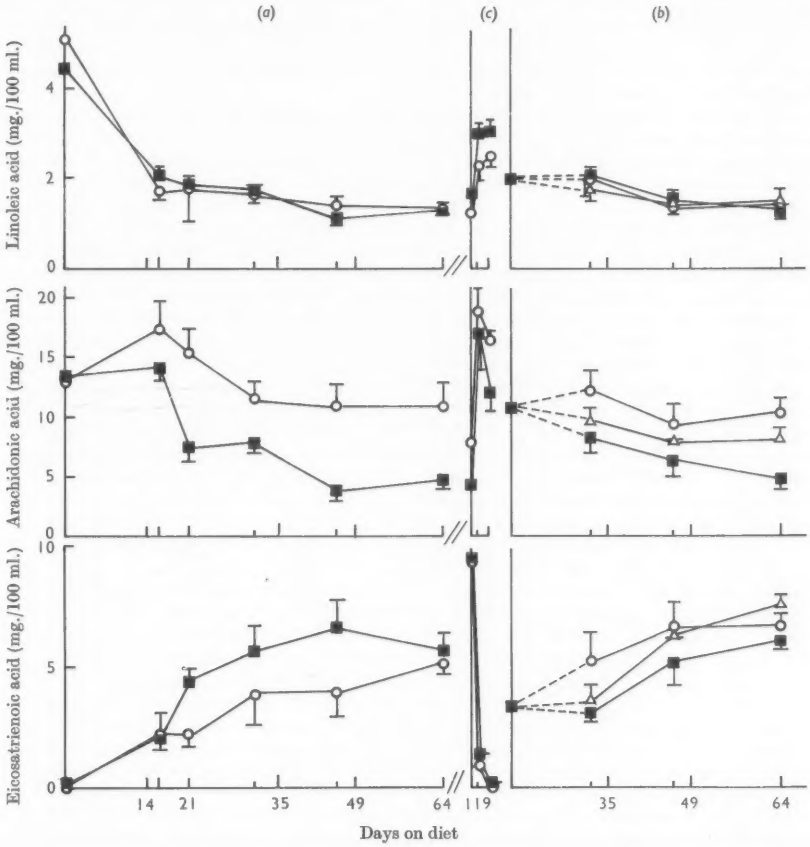

Fig. 3. Concentrations of linoleic acid, arachidonic acid and eicosatrienoic acid in plasma esterified cholesterol of rats during development of EFA deficiency. For explanation of symbols see Fig. 1.

acid and eicosatrienoic acid as the EFA deficiency progressed were similar to those for the phospholipids. Although the percentages of linoleic acid were the same for both sexes, females had higher proportions of arachidonic acid than males ($P < 0.02$ at 21, 46 and 64 days; $P < 0.05$ at 32 days). Oestradiol had a similar effect on arachidonic acid when administered to castrated rats. Males had significantly larger proportions of oleic acid ($P < 0.02$ at all periods) and eicosatrienoic acid ($P < 0.05$ at 32 days; $P < 0.01$ at 46 days). Testosterone significantly increased the proportion of oleic acid ($P < 0.05$ at 32 and 46 days; $P < 0.02$ at 64 days). However, no differences in eicosatrienoic acid resulted from hormone treatment.

The concentrations of linoleic acid, arachidonic acid and eicosatrienoic acid in the cholesteryl esters are shown in Fig. 3. Of these three fatty acids, only arachidonic acid showed significant sex differences. Females maintained significantly higher concen-

Table 4. *Effect of EFA deficiency in male, female and hormone-treated rats on the unsaturated fatty acids of plasma triglycerides*

Fatty acids are expressed as percentages of total methyl esters determined. Fatty acids above C_{20} in this fraction constituted less than 3% of the total. Means of the percentages or concentrations of fatty acids from five or more rats are shown.

			Castrated rats					
			Oil		Oestradiol		Testosterone	
Days on diet	Females (%, by wt.)	Males (%, by wt.)	(%, by wt.)	(mg./100 ml.)	(%, by wt.)	(mg./100 ml.)	(%, by wt.)	(mg./100 ml.)
				16:1				
0	1·6	2·2	—	—	—	—	—	—
16	8·0	7·7	—	—	—	—	—	—
21	6·5	7·7	—	—	—	—	—	—
32	7·6	8·8	8·1	(2·0)	7·1	(2·8)	7·0	(1·3)
46	8·6	8·4	8·8	(2·1)	6·8	(3·9)	7·2	(1·2)
64	7·6	8·5	8·2	(2·1)	7·0	(3·8)	8·0	(1·4)
				18:1				
0	16·6	19·0	—	—	—	—	—	—
16	39·1	38·2	—	—	—	—	—	—
21	39·9	44·4	—	—	—	—	—	—
32	40·6	41·8	40·8	(10·1)	45·2	(17·7)	37·3	(7·8)
46	40·6	39·9	40·1	(10·4)	47·1	(25·3)	38·4	(6·6)
64	40·3	37·7	43·3	(11·4)	47·1	(27·7)	42·0	(7·9)
				18:2				
0	23·6	24·8	—	—	—	—	—	—
16	6·1	6·8	—	—	—	—	—	—
21	6·7	4·1	—	—	—	—	—	—
32	3·8	3·2	4·3	(1·2)	3·5	(1·4)	4·3	(0·9)
46	4·3	3·5	2·7	(0·6)	2·5	(1·4)	4·0	(0·6)
64	4·9	4·3	2·7	(0·7)	2·5	(1·4)	3·1	(0·5)
				20:4				
0	10·5	14·0	—	—	—	—	—	—
16	2·5	2·9	—	—	—	—	—	—
21	3·4	0·3	—	—	—	—	—	—
32	1·6	1·4	0·8	(0·3)	1·4	(0·6)	1·7	(0·4)
46	2·1	0·8	2·0	(0·5)	1·8	(1·1)	3·1	(0·5)
64	1·2	0·8	1·0	(0·3)	1·3	(0·4)	1·2	(0·3)
				20:3				
0	—	—	—	—	—	—	—	—
16	2·8	3·8	—	—	—	—	—	—
21	5·1	4·5	—	—	—	—	—	—
32	5·8	5·3	5·3	(1·3)	5·4	(2·1)	6·1	(1·1)
46	4·6	5·1	6·7	(1·6)	6·8	(3·5)	6·7	(1·1)
64	5·7	4·4	4·8	(1·4)	4·9	(1·9)	5·5	(1·1)

trations than males ($P < 0.01$ at 21 and 46 days; $P < 0.05$ at 64 days). A less-pronounced effect on cholesteryl arachidonate was produced by oestradiol ($P < 0.01$ at 64 days only).

Although males consistently averaged more eicosatrienoic acid than females, any significant differences between them were eliminated by wide variations among the groups. Feeding again with linoleate (Fig. 3c) had an effect on linoleic acid, arachidonic acid and eicosatrienoic acid in the cholesteryl esters similar to that produced in the phospholipids. Quantitatively, the greatest changes were in arachidonic acid (two- to three-fold increase) and eicosatrienoic acid (six- to seven-fold decrease) after only a single day of feeding with linoleate. The principal saturated fatty acids associated with the cholesteryl esters (Table 2) were palmitic acid and stearic acid, the latter representing only about 3–4% of all the esterified fatty acids in this fraction. Unlike those of the phospholipids, the proportion and concentration of these fatty acids were not influenced by sex-hormone treatment or by EFA deficiency.

Triglyceride fatty acids. Plasma-triglyceride fatty acids are shown in Table 4. The proportions of linoleic acid and arachidonic acid decreased to very low values within the first 2 weeks of the deficiency, whereas those of oleic acid, palmitoleic acid and eicosatrienoic acid increased. No qualitative sex differences were found in this lipid fraction. However, as a result of the greatly increased triglyceride concentrations in the oestradiol-treated animals (Fig. 1), the concentrations of all the triglyceride fatty acids in this group were two or three times those of the other groups. The predominating fatty acids in this fraction were oleic acid and palmitic acid (Table 2). Quantitatively therefore these fatty acids appeared to increase more than any of the others, although proportionately all fatty acids increased about the same.

DISCUSSION

The relatively greater resistance of female than of male rats to EFA deficiency has been observed by many investigators (Loeb & Burr, 1947; Greenberg, Calbert, Savage & Deuel, 1950; Morton & Horner, 1961; Aftergood & Alfin-Slater, 1965). Ostwald *et al.* (1965) sought reasons for this sex difference by following the distribution of linoleic acid and arachidonic acid in the carcass and liver lipids of female and male rats during the development of EFA deficiency. The results showed that, although no sex differences in linoleate were evident, female rats had a higher proportion of arachidonic acid in their liver phospholipids than males. Total arachidonic acid in this lipid fraction (99% of liver arachidonic acid was in the phospholipids) was similar for both sexes, however. Males had larger livers, more liver phospholipid and more eicosatrienoic acid than the females. It was concluded that the need for more structural phospholipid by the faster-developing male rat necessitated synthesis of a phospholipid in which eicosatrienoic acid replaced the normal arachidonic acid. The early increase in concentration of this abnormal phospholipid in the tissues of the EFA-deficient male rat could account for the earlier appearance and more severe deficiency symptoms observed in these animals.

In the present study, plasma phospholipids and cholesteryl ester fatty acids from the same animals also revealed no sex differences in linoleic acid. Females, however, had significantly higher proportions and concentrations of arachidonic acid in both lipid fractions as compared with male rats. Therefore, during this experiment, in which the total liver linoleate and arachidonate were similar in both sexes, female rats maintained higher concentrations of arachidonic acid in plasma phospholipids and cholesteryl esters than did the males. It is generally recognized that plasma phospholipids are derived from the liver (Zilversmit & Bollman, 1956); therefore the higher proportions of arachidonic acid in the plasma phospholipids of females and eicosatrienoic acid in males would have resulted if both sexes secreted lipoproteins having similar proportions of the phospholipid sub-classes, because liver phospholipids of males had higher proportions of eicosatrienoic acid, whereas those of females maintained higher percentages of arachidonic acid. Comparison of the proportions of the liver-phospholipid fatty acids of both sexes (Ostwald *et al.* 1965) with their proportions in the plasma (Table 1) shows a considerable similarity in fatty acid composition in the two compartments. The small but consistent difference between plasma-phospholipid concentrations in male and female rats as the deficiency progressed would further accentuate the quantitative sex differences in the concentrations of the arachidonic acid (Fig. 1).

The absence of sex differences in proportion and concentration of linoleic acid as well as its low concentration in the lipid fractions of the EFA-deficient rats indicates that any linoleic acid available from adipose-tissue stores was converted into arachidonic acid so rapidly that little or no linoleic acid accumulated. The sudden increase in plasma phospholipid and cholesteryl arachidonate after only 1 day of feeding with linoleate (Figs. 2c and 3c) also illustrated the speed of the conversion and the avidity of these lipid fractions for the deficient fatty acid. The reciprocal decrease in eicosatrienoic acid in the phospholipid seems to support the contention of Brenner & José (1965) that arachidonic acid

competes with and replaces the eicosatrienoic acid in the phospholipids.

In general, the proportions of fatty acids in the plasma phospholipids of the oestradiol- and testosterone-treated rats were comparable with those of the intact female and male animals. In these EFA-deficient rats, however, oestradiol seemed to have a specific effect on plasma phospholipids (Fig. 1*b*), which elevated their concentrations to 1·5 times those of the other groups. Although the concentration of phospholipid was increased, the concentration of arachidonic acid did not rise above that of the intact female rat (Fig. 2*b*), whereas concentrations of eicosatrienoic acid, oleic acid and stearic acid, i.e. those fatty acids readily synthesized, increased. Therefore, when supplies of arachidonic acid are insufficient, synthesis of additional phospholipid seems to be accomplished by substituting eicosatrienoic acid for the unavailable arachidonic acid. Why oestradiol has such an effect on the plasma phospholipids of the EFA-deficient rats is not known. At the amounts administered, oestradiol maintained growth similar to that of the intact female rat, but the livers in these animals were enlarged to such an extent that they resembled the livers of males (Ostwald *et al.* 1965). The quantity of the hormone administered (30 μg./week) was chosen because, in castrated rats on normal diets, it maintained food consumption, growth and liver and adrenal weights at values comparable with those of intact female rats. Apparently EFA-deficient rats may have a greater sensitivity to oestrogen than have normal animals. Such a possibility has been discussed by Klein, Finerty, Panos & Wall (1957). In man, oestradiol increases serum high-density lipoproteins, which contain abundant quantities of phospholipid (Russ, Eder & Barr, 1955; Oliver & Boyd, 1958). Borden, Wissler & Hughes (1964) showed that, in the rat given high-fat diets and large doses of oestradiol (333 μg./day), serum low-density (β-) and high-density (α-) lipoproteins, both of which contained relatively large amounts of phospholipid, were greatly raised. Quantitatively, the increase in cholesterol concentration produced by the oestradiol was much less than the phospholipids. It may be that, in EFA-deficient rats, increased sensitivity to oestradiol causes a similar synthesis and secretion of phospholipid-rich lipoproteins. The increase in β-lipoprotein might also explain the rise in triglyceride induced in the castrated rats by oestradiol (Table 4).

EFA deficiency in rats developed by giving a fat-free diet has been reported to increase liver lipids and to decrease plasma cholesterol (Deuel, Alfin-Slater, Wells, Kryder & Aftergood, 1955), whereas the inclusion of hydrogenated coconut oil allows maintenance of normal liver and plasma cholesterol concentrations. In the present experiment, EFA deficiency was induced with diets containing 10% of hydrogenated coconut oil, so that neither liver-lipid accumulation nor changes in plasma cholesterol concentrations occurred. However, the higher concentrations of plasma cholesterol occurring in female and oestradiol-treated rats (Fillios, 1957; Okey & Lyman, 1956; Aftergood & Alfin-Slater, 1965) were not so obvious in these animals. The fatty acid composition of the plasma cholesteryl esters differed from the phospholipids principally by having higher proportions of palmitoleic acid and arachidonic acid and less palmitic acid and stearic acid. As with the plasma phospholipids, however, females had significantly higher proportions (Table 3) and concentrations (Fig. 3) of arachidonic acid than males. The sex differences in arachidonic acid in the esterified cholesterol fraction cannot be explained on the basis of a direct secretion from the liver to the plasma because the fatty acid composition of the plasma cholesteryl esters differs greatly from that of the liver esters (Klein, 1958; Okey *et al.* 1962; Aftergood & Alfin-Slater, 1965; Ostwald *et al.* 1965). Glomset (1962) suggested that the high proportion of polyunsaturated fatty acids in plasma cholesteryl esters arises from a plasma transesterification reaction between the β-unsaturated fatty acids of the plasma lecithins and free cholesterol. Sugano & Portman (1965) also consider that a reaction between plasma phospholipid and cholesterol, mediated by plasma enzymes, may play a significant role in the formation of plasma cholesteryl esters; but Roheim, Haft, Gidez, White & Eder (1963), using liver-perfusion techniques, and Goodman & Shiratori (1964), in turnover studies of individual cholesteryl esters *in vivo*, concluded that the liver was the major source of plasma esterified cholesterol. The greater proportion of arachidonic acid esterified with plasma cholesterol, as compared with the liver cholesteryl esters, has been reconciled by proposing that the liver selects, preferentially, polyunsaturated cholesteryl esters for lipoprotein formation (Goodman, Deykin & Shiratori, 1964; Swell, Law & Treadwell, 1964). In our experiment there was remarkable parallelism between the proportions and concentrations of arachidonic acid and eicosatrienoic acid in the plasma cholesteryl esters and phospholipids at each interval studied. Since these fatty acids occur almost exclusively on the β-position of the phospholipids (De Tomás, Brenner & Peluffo, 1963), apparently phospholipids are contributing their β-fatty acids directly for the formation of the plasma cholesteryl esters. Although the results suggest that this transesterification could take place in the plasma, the possibility of a selective secretion of cholesteryl arachidonate or eicosatrienoate from the liver cannot be dismissed without additional experimental evidence.

This study has shown that female and oestradiol-treated rats given a diet completely devoid of essential fatty acids could maintain, for relatively long periods, higher concentrations of plasma cholesteryl and phospholipid arachidonate than male or testosterone-treated animals. Much of this sex difference may be attributed to the smaller body and organ size of the female or oestrogen-treated rats. Less of their arachidonic acid therefore is fixed in the form of structural phospholipids, and more is allowed to be associated with the circulating lipids. We believe, however, that oestrogen may have a more specific effect on the metabolism of essential fatty acids than just suppression of growth. Lyman *et al.* (1963) showed that female or oestradiol-injected rats given more than adequate amounts of linoleic acid had higher concentrations of stearic acid and arachidonic acid in their plasma lecithins than males or testosterone- or oil-injected animals. Therefore, under conditions in which no shortage of arachidonic acid would be expected to occur in either sex, females still maintained higher concentrations of lecithin arachidonate. Tinoco, Shannon & Lyman (1964) demonstrated that, in choline-deficient rats, a condition in which phospholipid metabolism is undoubtedly affected, the sex differences in plasma phospholipid stearic acid and arachidonic acid were lost with reversion to a male pattern (i.e. to relatively higher proportions of palmitic acid and linoleic acid). In the EFA-deficient rats in this study, female characteristics were also associated with maintenance of a phospholipid that contained more stearic acid and arachidonic acid compared with deficient males or testosterone-treated rats. Collins (1960) and Harris, Robinson & Getz (1960) showed that lecithins containing high proportions of stearic acid and arachidonic acid are metabolically different from lecithins rich in palmitic acid and linoleic acid. Since female hormones have been associated with various aspects of phospholipid metabolism (Stekol, Weiss, Smith & Weiss, 1953; Bowser, Henderson & Zimmerman, 1961; Natori, 1963), it is possible that oestradiol may influence essential fatty acid metabolism through an effect on formation or maintenance of phospholipids characteristically rich in arachidonic acid.

The authors thank Miss Ruth Babcock for the numerous analytical determinations, and Mr Peter Miljanich for maintenance and standardization of the gas–liquid chromatographs. The assistance of Mr Tom Bouchard and Mrs Margaret Rose in care and handling of the animals is also greatly appreciated. This investigation was supported in part by U.S. Public Health Service Grant H-6480 and Western Regional Experiment Station Project W-44R. A preliminary report of this study has been presented at the Federation of American Societies for Experimental Biology, Chicago, Ill. (Lyman, Shannon & Ostwald, 1964*a*).

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Fluorescence Studies on some Hydroxypyridines including Compounds of the Vitamin B_6 Group

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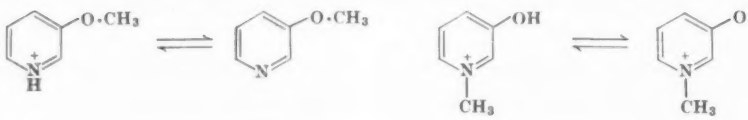
(*Received* 30 *July* 1965)

1. The variations with pH (from 36N-sulphuric acid to 10N-sodium hydroxide) of the excitation and fluorescence wavelengths and fluorescence intensity of 2-, 3- and 4-hydroxypyridine and their *O*- and *N*-methyl derivatives were investigated. 2. 4-Hydroxy- and 4-methoxy-pyridine were non-fluorescent at all pH values. 3. The cations and dipolar ions of the 3-hydroxypyridine derivatives and the anion of 3-hydroxypyridine were fluorescent, but the neutral forms were not. 4. All the forms of the 2-hydroxypyridine derivatives were fluorescent. 5. Pyridoxol, pyridoxal and its 5-phosphate, pyridoxamine and pyridoxic acid and its lactone were studied similarly. All these compounds, except pyridoxal 5-phosphate, were more fluorescent than 3-hydroxypyridine. 6. The most fluorescent forms of these compounds are the anions, except for pyridoxol, where the dipolar ion was the most fluorescent form. The least fluorescent forms are the neutral molecules. The dipolar ions were appreciably fluorescent in all cases. 7. The most fluorescent form examined was the dianion of pyridoxic acid lactone. 8. The cations were all fluorescent except the cations of 2- and 3-methoxypyridine. All the cations showed excited-state ionization. The excited pK_a values of these cations were determined and the results are discussed with reference to Weller's (1952) equation relating ground- and excited-state dissociation constants. 9. The pK_a values for all ionizations undergone by the compounds examined were determined from fluorescence data. 10. Stokes shifts for the various ionic and neutral species of the compounds examined were calculated and are discussed.

Although pyridine itself is non-fluorescent (Kerr, Hayes & Ott, 1957; Bridges, 1963), some of its biologically important derivatives such as pyridoxol, pyridoxal and pyridoxamine are fluorescent in aqueous solution at pH 7 (Duggan, Bowman, Brodie & Udenfriend, 1957). The fluorimetric determination of compounds of the vitamin B_6 group, however, is usually based on their conversion into the fluorescent pyridoxic acid lactone (e.g. Huff & Pealzweig, 1944; Reddy, Reynolds & Price, 1958; Fujita, Matsuura & Fujino, 1955; Fujita, Fujita & Fujino, 1955*a*,*b*), or into fluorescent cyanohydrins (Bonavita & Scardi, 1959; Polansky, Camarra & Toepfer, 1964). Compounds of the vitamin B_6 group possess native fluorescence, and in the present paper a study has been made of this fluorescence over a wide pH range. To interpret the fluorescence of these compounds, the fluorescence characteristics of 2-, 3- and 4-hydroxypyridine and their *O*- and *N*-methyl derivatives have also been examined. Some of the results described below have been briefly reported (Bridges, Creaven, Davies & Williams, 1964).

EXPERIMENTAL

Materials. 2-Hydroxypyridine, m.p. 107° (from benzene), 3-hydroxypyridine, m.p. 126° (by sublimation), 4-hydroxypyridine, m.p. 145° (as supplied), and 2-methoxypyridine, b.p. 142° (redistilled), were purchased (Koch–Light Laboratories Ltd., Colnbrook, Bucks.) and purified as indicated. 3-Methoxypyridine, b.p. 178–179° after redistillation, was prepared from 3-hydroxypyridine with ethereal diazomethane. 4-Methoxypyridine, b.p. 190° after redistillation, was prepared by heating 4-chloropyridine (m.p. 220°; Koch–Light Laboratories Ltd.) with sodium methoxide in a sealed tube at 100° for 1hr. 2-Hydroxy-*N*-methylpyridine hydrochloride, m.p. 166°, and the free base, m.p. 33°, and 3-hydroxy-*N*-methylpyridine hydrochloride, m.p. 158° after recrystallization from ethanol, were prepared from the hydroxypyridines with methyl iodide (Albert & Phillips, 1956). Pyridoxol (pyridoxine) hydrochloride, m.p. 205–206°, pyridoxal hydrochloride, m.p. 163° (decomp.), pyridoxamine hydrochloride, m.p. 226° (decomp.), pyridoxal 5-phosphate, and pyridoxic acid, m.p. 225° (decomp.), were purchased (Koch–Light Laboratories Ltd.) and purified where necessary. Pyridoxol was prepared from its hydrochloride (Stiller, Keresztesy & Stephens, 1939), and pyridoxal and pyridoxamine from their respective hydrochlorides (Metzler & Snell, 1955). Pyridoxic acid lactone



(I) (324 mμ) (II) (non-fluorescent) (III) (335 mμ) (IV) (395 mμ)

hydrochloride, m.p. 273–274°, was prepared from pyridoxic acid by a Fischer–Speirer esterification as described by Heyl (1948). A chromatographic examination of all the above compounds showed that they were free from impurities that might interfere with their fluorescence.

Preparation of solutions. Stock solutions were prepared by dissolving 0·1 g. of the appropriate compound in 100 ml. of glass-distilled water. With compounds sparingly soluble in water, e.g. 2- and 3-methoxypyridine, 20 ml. of ethanol (Burroughs RR grade) was added to assist solution. Stock solutions were diluted with glass-distilled water to the desired concentration. To obtain solutions of various pH values, the aqueous solutions were titrated with 0·001 N- to 10 N-HCl or -NaOH. pH values between 2 and 12 were measured in a Pye Master pH-meter. Solutions of pH values 0–2 and above 12 were prepared by adding HCl or NaOH of the appropriate strength and calculating the pH value on the assumption of complete ionization. The fluorescences of uncharged forms were determined with solutions of the free bases in ethanol. Fluorescence spectra were determined in the Aminco–Bowman spectrophotofluorimeter (American Instrument Co., Silver Springs, Md., U.S.A.) by using a 150 w Osram xenon lamp (XBO–165), and ultraviolet spectra in the Unicam SP.500 spectrophotometer.

All excitation and fluorescence wavelengths quoted in this paper are instrumental values.

Use of the acidity function. All the compounds tested for fluorescence in this paper have been examined in solutions of very high acidity, i.e. 1–36 N. For these solutions it has been necessary to express high acidity in terms of the acidity function, H_0, of Hammett (1940) rather than use negative pH, which is merely a theoretical extension of the normal pH scale. H_0 is a practical measurement of the ability of the medium to protonate an uncharged base. Normality or concentration of the acid and H_0 are related and the values of H_0 used for HCl and H_2SO_4 are those of Paul & Long (1957). At pH 0 and above, H_0 and pH are virtually the same (see Albert & Serjeant, 1962).

Stability of compounds in ultraviolet light. 3-Hydroxypyridine was stable in aqueous solutions (5 μg./ml.; pH 5·8) when exposed for up to 5 hr. to light of 330 mμ from the xenon lamp of the fluorimeter. However, pyridoxol, pyridoxal and pyridoxamine photodecomposed under these conditions, their fluorescence intensities falling to half the initial values at 40, 80 and 22 min. respectively. Fluorescence-intensity readings were made within 1 min. of exposure to the exciting light.

Effect of concentration on fluorescence intensity. All the compounds studied showed some concentration quenching above 5 μg./ml. The solutions were therefore examined at concentrations of about 5 μg./ml. or less, where fluorescence intensity was proportional to concentration.

Correction of intensity values. In Tables 1–4 there are quoted observed and corrected values for fluorescence intensity. In the calculation of these values, the intensity of fluorescence of the 3-hydroxypyridine anion at pH 11 has been given the arbitrary value of 100 and all other intensities were based on this, making allowances for molecular weight. Observed values were calculated from the photomultiplier readings of the fluorimeter, whereas for the corrected values allowance was made for the variation in energy output of the xenon lamp, which was calibrated by the chemical actinometer method of Hatchard & Parker (1956).

Estimation of pK *values.* pK_a values were determined where possible from the fluorescence-intensity, excitation-wavelength and/or fluorescence-wavelength curves, which were drawn out in detail. The pH at which a compound fluoresced with 50% of the maximum intensity was taken as the pK_a. This was done when the intensity curve obtained was suitable for the purpose. With excitation- and fluorescence-wavelength curves, the pK_a was taken as the pH at the half-way point in a wavelength change when this change was of suitable magnitude.

RESULTS AND DISCUSSION

Wavelengths given in parentheses under formulae in the succeeding pages are those of the maximum fluorescence of the compound.

3-Hydroxypyridine and its methyl ethers. The fluorescence of 3-methoxypyridine is relatively simple. Only two molecular species are involved, namely the cation (I), which fluoresces at 324 mμ ($\lambda_{exc.}$ 295 mμ), and the neutral molecule (II), which is non-fluorescent. The cation (I) has a maximum fluorescence intensity at about pH 1·9–3·3, and as the pH rises above this the intensity falls to zero at pH 7 (see Fig. 1), the fall following the ionization curve of the cation (I). The pK_a calculated from the intensity curve is 4·9, which is in agreement with the value 4·88 (Albert & Phillips, 1956) obtained by other methods (see Table 1). Below pH 2, the fluorescence intensity diminishes and disappears in 10 N-hydrochloric acid ($H_0 - 3·7$) and this may be due to quenching by Cl^- or H^+ ions. Whereas the cation (I) ionizes by loss of a proton from the ring nitrogen atom, 3-hydroxy-*N*-methylpyridine (III) ionizes by loss of a proton from the hydroxyl group, forming the dipolar ion (IV). Both species (III and IV) are fluorescent but the fluorescence of the cation (III) is observable only below

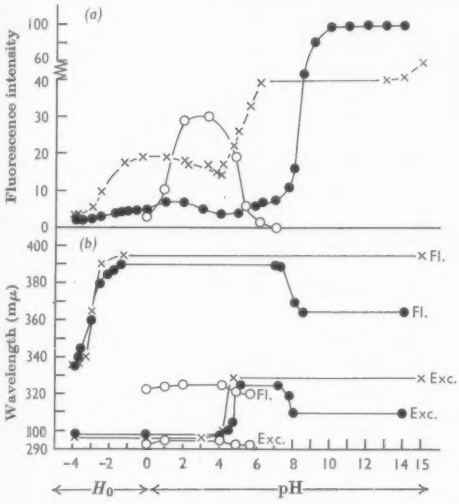


Fig. 1. Variation of (*a*) fluorescence intensity and of (*b*) fluorescence (Fl.) and excitation (Exc.) wavelengths with pH and H_0 of 3-hydroxypyridine (●—●), 3-methoxypyridine (○—○) and 3-hydroxy-*N*-methylpyridine (×—×) (concentration of each 5 μg./ml.). H_0 is the acidity function of Hammett (see the text). Intensity values are arbitrary, that of the 3-hydroxypyridine anion at pH 11 being taken as 100.

$H_0 - 3$ (8N-hydrochloric acid) at 335mμ ($\lambda_{exc.}$ 298mμ). This cation shows excited-state ionization, for when the acidity function is raised from $H_0 - 4{\cdot}15$ to $-1{\cdot}05$ (11·6N- to 3N-hydrochloric acid) the fluorescence wavelength gradually increases to that of the dipolar ion (IV) (395mμ), but the excitation wavelength (298mμ) remains the same until pH about 4·5 (see Fig. 1). In this pH region, the excitation gradually changes with pH to 328mμ, but the fluorescence remains at 395mμ, and the change in excitation follows the normal ionization of the cation (III) (see Table 1 and Fig. 1). The fluorescence wavelength of 3-hydroxy-*N*-methylpyridine is thus constant from $H_0 - 1{\cdot}05$ to pH 14, but the intensity curve shows changes in the regions $H_0 - 3{\cdot}7$ to $-0{\cdot}2$ and pH 4–6, the first corresponding to the excited-state ionization and the second to the normal ionization of the cation (III). The dipolar ion (IV) is more fluorescent than the cation (III). At pH 14, the fluorescence intensity of 3-hydroxy-*N*-methylpyridine increases, possibly owing to slight demethylation to the highly fluorescent 3-hydroxypyridine anion (VIII), as suggested by the wavelengths of excitation and fluorescence (Table 1).

3-Hydroxypyridine can exist in solution at various pH values in four forms, namely the cation (V), dipolar ion (VI), neutral molecule (VII) and anion (VIII). The above observations on the methyl ethers suggest that the neutral molecule (VII) is unlikely to be fluorescent, and in ethanol 3-hydroxypyridine is, in fact, virtually non-fluorescent. In aqueous solution, the two forms occur in the proportion of 54% of the dipolar ion (VI) to 46% of the neutral molecule (VII) (Metzler & Snell, 1955). The 3-hydroxypyridine cation (V), like its *N*-methyl derivative (III), shows excited-state ionization. The fluorescence of the cation (V) occurs at about 340mμ and is observable only at $H_0 - 3{\cdot}7$ to $-3{\cdot}25$. Above the latter H_0, the fluorescence wavelength changes to that of the dipolar ion (392mμ), which persists to pH 7. The excitation wavelength (283mμ), however, remains unchanged to pH 4, and then changes gradually to 325mμ over the pH range at which normal ionization occurs. The pK_a calculated from the excitation curve is 4·80 (see Table 1). Both excitation and fluorescence wavelengths change again at pH 7–8, owing to ionization to the anion (VIII). The pK_a for this ionization, estimated from the fluorescence-intensity curve, is 8·5 (see Table 1). The intensity curve (see Fig. 1) shows that the cation and dipolar ion are not strongly fluorescent. The dipolar ion (VI) fluoresces maximally at about $H_0 - 0{\cdot}2$ to pH 3·0, but this is followed by a slight fall in intensity between pH 3 and pH 5, which is probably due to the formation of varying amounts of the non-fluorescent neutral form (VII). At pH 6 the intensity begins to increase slowly, then rapidly at pH 8, until at pH 11 the intensity reaches a maximum due to the highly fluorescent anion (VIII).

The neutral molecules (II and VII), not unexpectedly (see Williams & Bridges, 1964), appear to be non-fluorescent. The neutral form of 3-hydroxyquinoline, which is a comparable form, is also non-fluorescent in water (Haylock, Mason & Smith, 1963).

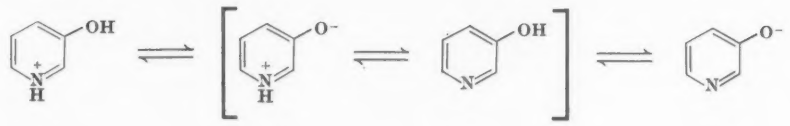
2-Pyridone and its methyl ethers. The fluorescence of 2-methoxypyridine (see Fig. 2) is similar to that of its 3-isomer. The cation (IX) fluoresces at 325mμ with a maximum intensity confined to a very narrow pH range (about 2·5). Above this the intensity falls with increasing pH, and follows the ionization curve closely. The pK_a, estimated from the intensity curve, is 3·2, which compares well with the value 3·28 (Albert & Phillips, 1956) obtained by other methods. Between pH 2·5 and pH 4·2, the excitation and fluorescence wavelengths change and at pH 4·2 the maximum fluorescence wavelength is 315mμ, which is that of the neutral molecule (X). The fluorescence of the neutral molecule (X) is, however, relatively weak but persists to pH 14. Below pH 2 the fluorescence of the

Table 1. *Fluorescence of 3-hydroxypyridine and its methyl ethers*

The concentration of each compound was 5 μg./ml.

Compound	Molecular species	Wavelength (mμ) Of max. absorption*	Of max. excitation	Of max. fluorescence	Relative fluorescence intensity‡ Observed	Corrected§	pH range of max. fluorescence	pK_a Int.‖	Exc.‖	Fl.‖	Literature values
3-Methoxypyridine	Cation	284	295	324	30·2	39·0	pH2·5–3·5	4·9	—	—	4·88¶
	Neutral form	276	—	None	0·0	0·0					
3-Hydroxy-*N*-methyl-pyridine	Cation	288	298	335	3·0	3·8	about H_0-4	5·1	4·4	—	4·96¶
	Cation (excited)	—	298	395	18·3	23·0	H_0 −0·3 to pH1·0	—	—	−3·0**	
	Dipolar ion	320	328	395	38·4	28·6	pH6–14				
3-Hydroxypyridine	Cation	283	298	about 340	2·0	2·5	about H_0-4	—	4·8	—	4·86¶, 5·10††
	Cation (excited)	—	298	392	7·1	8·9	pH1–2	—	—	−3·0**	
	Dipolar ion	315	325	392	6·1	4·7		8·5	7·9	7·85	8·60††, 8·72‡‡
	Neutral form	278†	—	None	0·0	0·0					
	Anion	298	309	365	100·0	100·0	pH10–14				

* Mason (1959).
† In ethanol (Mosher, 1950).
‡ Intensity of 3-hydroxypyridine anion at pH 11 = 100.
§ See the text.
‖ pK_a values estimated from intensity curve (Int.), or excitation-wavelength change (Exc.), or fluorescence-wavelength change (Fl.) where convenient (see Fig. 1).
¶ Albert & Phillips (1956).
** Excited-state pK_a (see the text).
†† Metzler & Snell (1955).
‡‡ Albert & Hampton (1954).



(V) (340mμ) (VI) (392mμ) (VII) (non-fluorescent) (VIII) (365mμ)

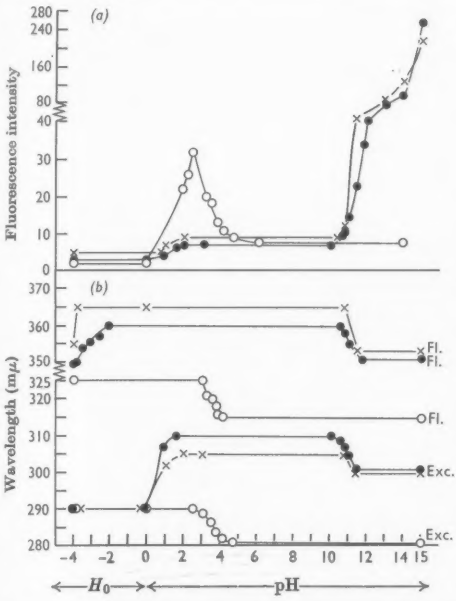


Fig. 2. Variation of (*a*) fluorescence intensity and of (*b*) fluorescence (Fl.) and excitation (Exc.) wavelengths with pH and H_0 of 2-hydroxypyridine (●—●), 2-methoxypyridine (○—○) and 2-hydroxy-*N*-methylpyridine (×—×) (concentration of each 5μg./ml.). H_0 is the acidity function of Hammett (see the text). Intensity values are arbitrary, that of the 3-hydroxypyridine anion at pH 11 being taken as 100.

(IX) (325mμ) (X) (315mμ)

cation (IX) diminishes to a small value, which is still observable in 10N-hydrochloric acid ($H_0 - 3{\cdot}7$). This fall in fluorescence may be due to quenching by Cl^- or H^+ ions.

The possible forms of *N*-methyl-2-pyridone occurring in solution at various pH values are structures (XI)–(XIV). The cation could be (XI) or the protonated pyridone (XII) (Albert & Phillips, 1956). Because of excited-state ionization, the fluorescence of the cation (345mμ) is observable only in concentrated acid (36N-sulphuric acid). The fluorescence wavelength changes at about $H_0 - 3{\cdot}8$ to 365mμ, whereas the excitation remains at 290mμ until $H_0 - 0{\cdot}3$ (1·2N-hydrochloric acid) is reached. The excitation then changes over the range $H_0 - 0{\cdot}3$ to pH 2·0, where structure (XI) [or (XII)] ionizes normally to structure (XIII) [or (XIV)]. The pK_a for this change, estimated from the excitation curve, is about 0·5 (see Table 2). It seems probable that the fluorescence at 365mμ is due to structure (XIII) resonating with structure (XIV). Above pH 10·8, the fluorescence and excitation wavelengths shorten to the values for the anion of 2-pyridone, probably because of alkaline demethylation of *N*-methyl-2-pyridone.

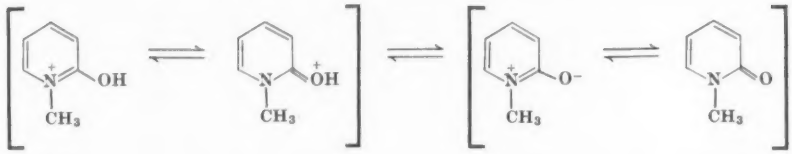
2-Pyridone shows fluorescence characteristics that are similar to its *N*-methyl derivative (see Fig. 2). The cation (XV) (pyridone form not shown here) shows excited-state ionization and its fluorescence (345mμ) is detectable only in 36N-sulphuric acid. The fluorescence wavelength changes to 360mμ at $H_0 - 1{\cdot}95$ (5·5N-hydrochloric acid), but the excitation does not change until pH about 0 is reached. The excitation change occurs where the cation (XV) ionizes normally to structure (XVI) [and (XVII)]. The pK_a of this change, estimated from the excitation-wavelength curve, is 0·6 (see Table 2). There are two changes in the intensity curve (see Fig. 2), one at pH 0 and the other at pH 10, these corresponding to the normal ionizations of (XV) to (XVI) [and (XVII)] and of (XVI) to (XVIII) [and (XIX)] respectively. The fluorescence at 360mμ is probably due to the dipolar ion (XVI) in resonance with the pyridone (XVII). In ethanol, the pyridone (XVII) fluoresces maximally at 370mμ ($\lambda_{exc.}$ 320mμ). At pH about 11 and above, 2-pyridone becomes intensely fluorescent, the wavelength of which shortens to 351mμ ($\lambda_{exc.}$ 300mμ). This is presumably due to the formation of the anion (XVIII), which is more than twice as

Table 2. *Fluorescence of 2-hydroxypyridine and its methyl ethers*

The concentration of each compound was 5 μg./ml.

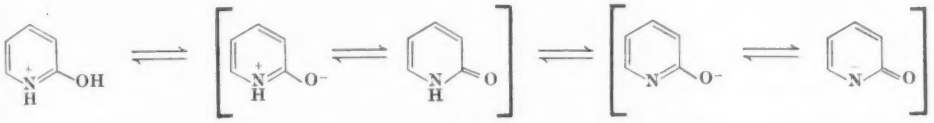
Compound	Molecular species	Wavelength (mμ) Of max. absorption*	Of max. excitation	Of max. fluorescence	Relative fluorescence intensity‡ Observed	Corrected	pH range of max. fluorescence	pK_a Int.§	Exc.§	Fl.§	Literature values
2-Methoxypyridine	Cation	279	290	325	32·1	48·8	pH2·5 (peak)	3·2	3·65	3·4	3·28¶
	Neutral form	269	281	315	6·9	13·2	pH6–14				
N-Methyl-2-pyridone	Cation	279	290	345	4·8	7·3	H_0 − 8·6	—	0·5	—	0·32¶
	Cation (excited)	—	290	365	5·3	8·2	H_0 − 4 to pH0	—	—	←4·0‖	
	Dipolar ion and/or pyridone	297	305	365	9·0	9·2	pH2–10·4				
	Anion by demethylation?	(291)	300	352	—	—	(Occurs in alkaline solution)				
2-Hydroxypyridine	Cation	277	290	345	4·3	6·5	H_0 − 8·6	—	0·6	—	0·75¶
	Cation (excited)	—	290	360	4·3	6·5	H_0 − 4 to pH0	—	—	−3·3‖	
	Dipolar ion and/or pyridone	293	310	360	8·6	8·2	pH2–10	—	11·0	11·1	11·62¶
	Anion	291	300	351	258·7	215·2	about pH15 (10N-NaOH)				
	Pyridone	300†	320	370	—	—	(In ethanol)				

* Mason (1959).
† In ethanol (Mosher, 1950).
‡ 3-Hydroxypyridone anion at pH 11 = 100.
§ See Table 1.
‖ Excited-state pK_a.
¶ Albert & Phillips (1956).



(XI) (XII) (XIII) (XIV)

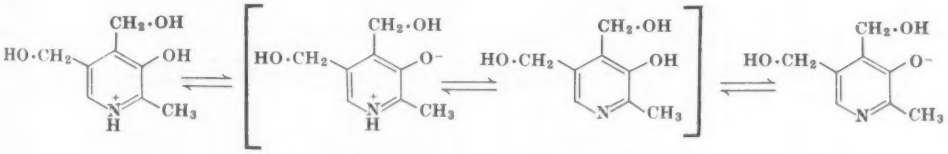
(345 mμ) (365 mμ)



(XV) (XVI) (XVII) (XVIII) (XIX)

(345 mμ) (360 mμ) (351 mμ)

(XX) (XXI)



(XXII) (XXIII) (XXIV) (XXV)

(345 mμ) (400 mμ) (335 mμ in methanol) (380 mμ)

fluorescent as the corresponding anion of 3-hydroxypyridine (see Table 2). It is possible for two anions to exist, namely (XVIII) and (XIX), the second being formed by deprotonation of the nitrogen atom of the pyridone (XVII). The existence of the anion (XIX) may explain the high fluorescence of 2-pyridone in alkaline solution.

4-Hydroxypyridine. Neither 4-hydroxy- nor 4-methoxy-pyridine was fluorescent at any pH. 4-Hydroxypyridine occurs in aqueous solution almost entirely as the pyridone (XX), which is non-aromatic (Albert & Phillips, 1956). This could explain its lack of fluorescence. 4-Methoxypyridine (XXI), like its 3-isomer, is non-fluorescent and, presumably, the methoxyl group in the 3- or 4-position is not sufficiently powerful to counteract the deactivating effect of the pyridine nitrogen atom. 2-Methoxypyridine, as shown above, is weakly fluorescent.

Pyridoxol. This compound fluoresces like 3-hydroxypyridine, for fluorescence maxima can be distinguished for the cation (XXII), dipolar ion (XXIII) and anion (XXV). Each of these species is more fluorescent than the 3-hydroxypyridine anion, the decreasing order of fluorescence intensity being: (XXIII), (XXV), (XXII). The cation also

Table 3. *Fluorescence of pyridoxol and its derivatives*

The concentration of each compound was 5μg./ml.

Compound	Molecular species	Wavelength (mμ) Of max. absorption*	Of max. excitation	Of max. fluorescence	Intensity of maximum fluorescence† Observed	Corrected	pH range of maximum fluorescence	pK_a Int.‡	Exc.‡	Fl.‡	Literature values
Pyridoxol (pyridoxine)	Cation	291	302	345	7·5	10	H_0 −3·7 to −2·8	5·0	4·7	—	5·0‖¶
	Cation (excited)	—	302	400	130	150	pH2·5	—	—	−2·2§	
	Dipolar ion	324	332	400	238	165	pH6·5–7·5	—	9·6	9·4	8·96¶, 8·97‖, 8·72
	Anion	310	320	380	168	144	pH12–14				
	Neutral form	286	296	335	—	—	(In ethanol or dioxan)				
Pyridoxal hemiacetal	Cation	288	300	350	11	13	H_0 −3·7 to −3·4	3·8	3·9	—	4·20*, 4·23¶
	Cation (excited)	—	300	382	125	145	pH0	—	—	−3·3§	
	Dipolar ion	317	330	382	207	150	pH6	—	8·9	9·0	8·66*, 8·70¶
	Anion	302	310	365	283	270	pH12				
	Neutral form	280 (dioxan)	291	330	—	—	(In ethanol)				
Pyridoxamine	Dication	292	306	350	70	95	H_0 −4·9	3·0	2·8	—	3·31*, 3·4‖, 3·54¶
	Dication (excited)	—	302	400	325	365	H_0 −1·0 to −0·8	—	—	−4·1§	
	Tripolar ion	326	337	400	370	245	pH4·9–5·5	8·2	9·4	9·6	7·90*, 8·21¶
	Dipolar ion		—	None	0	0					
	Anion	310	320	370	410	345	pH15				
	Neutral form	287 (dioxan)	309	361	—	—	(In ethanol)				
Pyridoxal 5-phosphate	Cation	291	302	385	<1·0	<1·0	H_0 −3·5				

Pyridoxal 5-phosphate aldehyde hydrate	Cation	291	302	385	<1·0	<1·0	H_0−2·5	—	—	2·3	<2·5¶
	Cation (excited)	—	302	400	11	13	pH1				
	Cation–phosphate monoanion	291	302	400				—	3·7	—	4·14¶
	Cation–phosphate monoanion (excited)	—	302	410							
	Dipolar ion–phosphate monoanion	324	320	410	11	8	pH6	—	—	6·5	6·20¶
	Dipolar ion–phosphate dianion	324	330	400			pH7·5–8·5	—	8·9	8·75	8·69¶
	Trianion	305	315	370	17·5	20·5	pH12–14				
	Trianion aldehyde form? .	388	400	525	about 2·0	—	pH12–14				

* Metzler & Snell (1955).
† 3-Hydroxypyridine anion at pH11 = 100; see the text and Table 1.
‡ See Table 1.
§ Excited-state pK_a.
‖ Lunn & Morton (1952).
¶ Williams & Neilands (1954).

Fig. 3. Fluorescence intensity of pyridoxol (5 μg./ml.) in ethanol–water mixtures: ●—●, dipolar ion, $\lambda_{fl.}$ 400 mμ ($\lambda_{exc.}$ 332 mμ); ×—× neutral molecule, $\lambda_{fl.}$ 335 mμ ($\lambda_{exc.}$ 296 mμ).

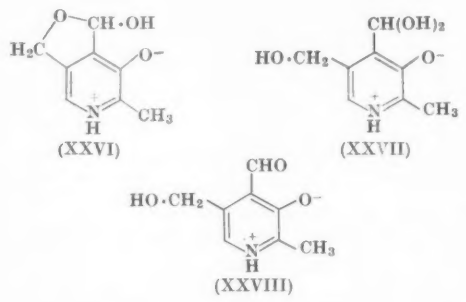
Fig. 4. Variation of (*a*) fluorescence intensity and of (*b*) fluorescence (Fl.) and excitation (Exc.) wavelengths with pH and H_0 of pyridoxine (○—○) and pyridoxal (●—●) (concentration of each 5 μg./ml.). H_0 is the acidity function of Hammett (see the text). Intensity values are arbitrary, that of the 3-hydroxypyridine anion at pH 11 being taken as 100.

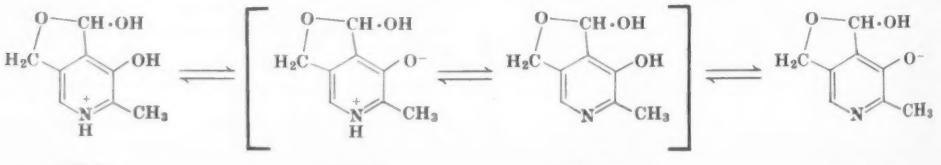
shows excited-state ionization. The neutral molecule (XXIV) shows a weak fluorescence in ethanol, and in mixtures of ethanol and water the fluorescence of structure (XXIII) or (XXIV) or both can

be detected according to the concentration of ethanol. In water it fluoresces as structure (XXIII), but in pure ethanol as structure (XXIV) (see Fig. 3). The true fluorescence of the cation (XXII) is detectable in 6–10·05N-hydrochloric acid ($H_0 - 3{\cdot}0$ to $-3{\cdot}8$), for above $H_0 - 3{\cdot}0$ the cation (XXII) shows excited-state ionization (see Table 3 for pK_a and fluoresces as structure (XXIII). This fluorescence persists from $H_0 - 3{\cdot}0$ to pH 9·0 (see Fig. 4), but the excitation corresponding to structure (XXII) persists to pH 4·3 and then changes to that of structure (XXIII) at pH 5. The pK_a of the ionization of the cation (XXII) is reported as 5·0 (see Table 3), whereas the value estimated from the excitation wavelength curve is 4·7 (see Fig. 4). Excitation and fluorescence wavelengths change together at pH 9·0–10·5 owing to formation of the anion (XXV). The pK_a for this ionization, estimated from the fluorescence- and excitation-wavelength curves (Fig. 4), are 9·4 and 9·6 respectively (see Table 3). The anion (XXV) is less fluorescent than the dipolar ion (XXIII), which is the reverse of the relationship for the corresponding species of 3-hydroxypyridine, and the most fluorescent form of pyridoxol is the dipolar ion (XXIII), at pH 7. The more intense fluorescence of the various forms of pyridoxol, as compared with 3-hydroxypyridine, may be due to the electron contribution of the methyl and hydroxymethyl groups (see Bridges & Williams, 1962). The dipolar anion (XXIII) shows two peaks of fluorescence intensity, namely at pH 2·5 and 7·0, the latter being about twice as intense as the former. The probable explanation for this is that at pH 2·5 the excitation is at 302mμ whereas at pH 7 it is at 332mμ. At 330mμ the energy output of the xenon lamp is greater than at 300mμ and, further, the efficiency of the absorption of energy by the cation followed by emission of energy as the dipolar ion is less than that in which the dipolar ion is both the absorbing and emitting species.

Pyridoxal. The fluorescence characteristics of pyridoxal at various pH values are shown in Fig. 4, and the pattern is similar to that of 3-hydroxypyridine, except that the latter is less fluorescent. Pyridoxal could exist in solution as a cyclic hemiacetal (XXVI), an aldehyde hydrate (XXVII) and a free aldehyde (XXVIII). These forms are distinguishable by absorption spectra, and Metzler & Snell (1955) have shown that the cyclic hemiacetals [e.g. (XXVI)] are the predominant forms in solution. The excitation wavelengths for the cation (300mμ), dipolar ion (330mμ), neutral molecule (291mμ) and anion (310mμ) correspond to the absorption data for the hemiacetal forms (Table 3) and therefore the fluorescences found are those of the hemiacetal forms (XXIX), (XXX), (XXXI) and (XXXII). These experiments suggest that each of the four forms (XXIX)–(XXXII) occurs, or occurs mainly, as a hemiacetal. The aldehyde group tends to diminish the fluorescence of aromatic systems (Bridges & Williams, 1962) because of its tendency to withdraw electrons from the ring. If pyridoxal occurred in the aldehyde form, then its fluorescence would probably be less intense than is found.

The fluorescence of the cation (XXIX) is found only in concentrated acid, since it shows excited-state ionization (see Fig. 4). The most fluorescent form of pyridoxal is the anion (XXXII) (at pH 12), which is more than 2·5 times as intense as the 3-hydroxypyridine anion (see Table 3). The fluorescence of the dipolar ion (XXX), which is 35 times as intense as that of the dipolar ion of 3-hydroxypyridine, shows two peaks of intensity, one at pH 0 and the other at pH 6 (cf. pyridoxol). The intensity





curve (Fig. 4) also shows an unexplained sharp peak at pH 8·5 that was repeatedly found. The rise in intensity at pH 8·8 is due to the formation of the anion (XXXII), and the fall in intensity above pH 12 could be due to some chemical change in alkali, possibly with the formation of the free aldehyde.

The pK_a values for pyridoxal determined from our fluorescence data are comparable with those reported in the literature (see Table 3).

Pyridoxamine. Four forms of pyridoxamine occur at various pH values in aqueous solution, namely the dication (XXXIII), tripolar ion (XXXIV), dipolar ion (XXXV) and anion (XXXVI). Fluorescence peaks were found that corresponded to forms (XXXIII), (XXXIV) and (XXXVI) (see Table 3 and Fig. 5) but not to (XXXV). No fluorescence peak was found for the neutral form (XXXVII) in aqueous solution, but in ethanol it fluoresced at 361 mμ. Because the cation (XXXIII) shows excited-state ionization its fluorescence was found only at $H_0 - 4{\cdot}9$ (20N-sulphuric acid). The wavelength of fluorescence changes to that of structure (XXXIV) at $H_0 - 1$ (2·9N-hydrochloric acid) and this persists to pH 9·3. The excitation, however, changes at pH 2·6–3·0 and the pK_a estimated from this change is 2·8 (see Table 3). At pH about 9, the excitation and fluorescence wavelengths shorten together and they correspond to a change from structure (XXXIV) to (XXXVI). The pK_a for this change estimated from the excitation and fluorescence wavelengths is 9·4–9·6. This value is about half-way between the reported pK_a values for the ionizations of (XXXIV) to (XXXV) and of (XXXV) to (XXXVI), which are given by Williams & Neilands (1954) as 8·21 and 10·63 respectively. Again at pH 9·4, the fluorescence intensity falls to a low value. These observations suggest that the dipolar ion (XXXV) is virtually non-fluorescent. This could be due, in simple terms, to the combined effect of the heterocyclic nitrogen atom and the charged $CH_2 \cdot NH_3^+$ group in deactivating the ring, and to interaction between the O^- group and the positively charged amino group, so that the ring-activating effect of O^- is diminished. The anion (XXXVI) is fluorescent with about the same intensity as the tripolar ion (XXXIV). Most of the fluorescent forms of pyridoxamine are more than three times as fluorescent as the 3-hydroxypyridine anion.

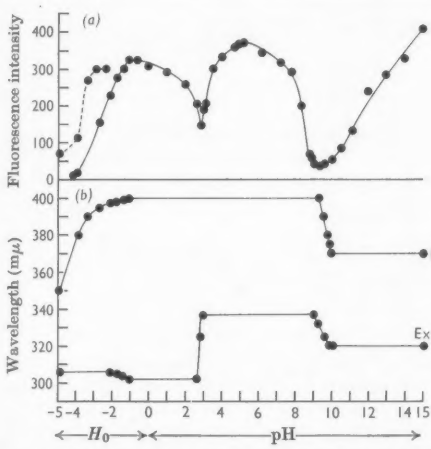
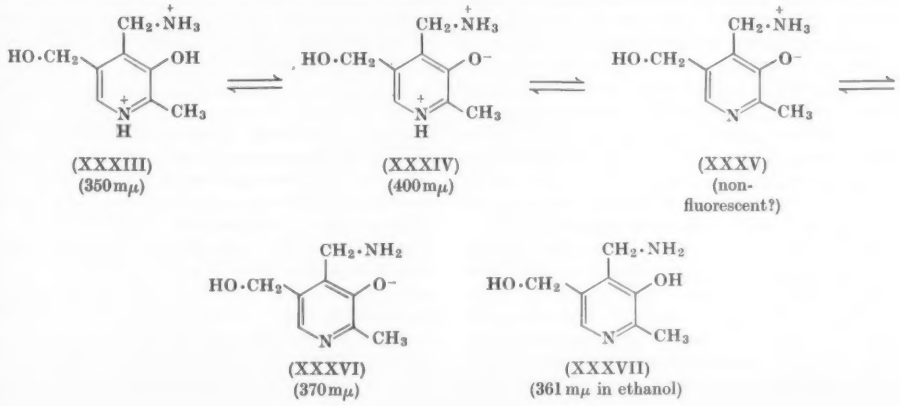


Fig. 5. Variation of (*a*) fluorescence intensity and of (*b*) fluorescence (Fl.) and excitation (Exc.) wavelengths with pH and H_0 of pyridoxamine (5 μg./ml.). H_0 is the acidity function of Hammett (see the text). Intensity values are arbitrary, that of the 3-hydroxypyridine anion at pH 11 being taken as 100. The broken line shows intensities measured in H_2SO_4.



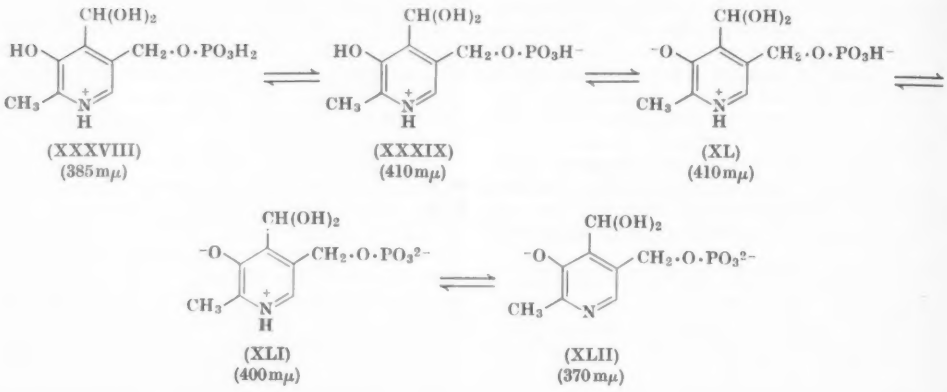


Fig. 5 shows also the effect of concentrated sulphuric acid and hydrochloric acid on the fluorescence of the pyridoxamine dication, which is more intense in sulphuric acid than in hydrochloric acid. This suggests that its fluorescence is more readily quenched by the latter.

Pyridoxal 5-phosphate. The fluorescence characteristics of this compound are shown in Fig. 6. It is immediately apparent that pyridoxal 5-phosphate is but weakly fluorescent, being only about 10% as fluorescent as the 3-hydroxypyridine anion. According to Williams & Neilands (1954), pyridoxal phosphate (aldehyde hydrate form) shows four pK_a values, namely $<2{\cdot}5$, 4·14, 6·20 and 8·69, which correspond to the equilibria between structures (XXXVIII)–(XLII). The curves shown in Fig. 6 contain evidence for all these changes. The wavelength shift in the fluorescence from 400 to 410mμ at pH 2·0–2·5 suggests the conversion of (XXXVIII) into (XXXIX), the wavelength shift in excitation from 302 to 331mμ at pH 3·0–4·6 suggests the conversion of (XXXIX) into (XL), the wavelength shift in fluorescence from 410 to 400mμ at pH 6·0–7·5 suggests the conversion of (XL) into (XLI), and the wavelength shifts in excitation (331 to 315mμ at pH 8·5–10·0) and in fluorescence (400 to 370mμ at pH 8·5–10·0) suggest the conversion of (XLI) into (XLII). These changes are also supported by fluorescence-intensity changes. Approximate pK_a values can be estimated from these changes and they are evaluated as 2·3, 3·7, 6·5 and 8·8 (see Table 3).

The cation (XXXVIII) shows excited-state ionization, since its true fluorescence wavelength maximum is in the region of 385mμ, which shows up at $H_0 - 2{\cdot}5$ (6·7N-hydrochloric acid). When the cation (XXXVIII) undergoes excited-state ionization it probably forms the dipolar ion (XLIII), in which the phosphate group is not ionized and which

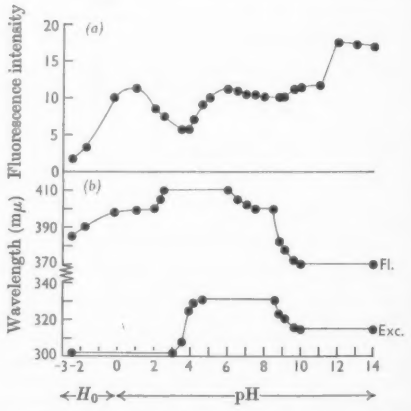
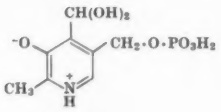
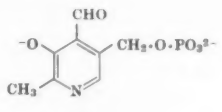


Fig. 6. Variation of (*a*) fluorescence intensity and of (*b*) fluorescence (Fl.) and excitation (Exc.) wavelengths with pH and H_0 of pyridoxal 5-phosphate (5μg./ml.). H_0 is the acidity function of Hammett (see the text). Intensity values are arbitrary, that of the 3-hydroxypyridine anion ion at pH 11 being taken as 100.

fluoresces at 400mμ. It appears also that structure (XXXIX) (cation–monophosphate anion) undergoes excited-state ionization, for at pH 2·5 the fluorescence (410mμ) of structure (XL) appears whereas the corresponding excitation does not change completely until pH 4·6 has been reached. The change from structure (XL) to (XLI) involves a small change in fluorescence but not in excitation wavelength, and in fact structures (XL) and (XLI) have the same maximum absorption peaks (Metzler & Snell, 1955). The conversion of structure (XLI) into (XLII) involves changes in fluorescence and



(XLIII)
(400mμ)



(XLIV)
(525mμ?)

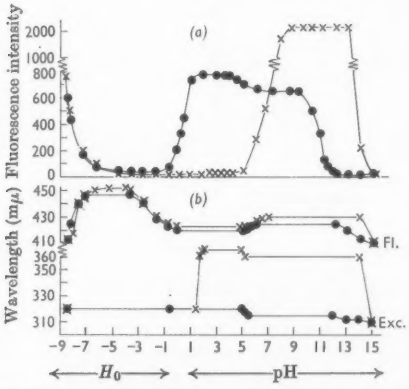


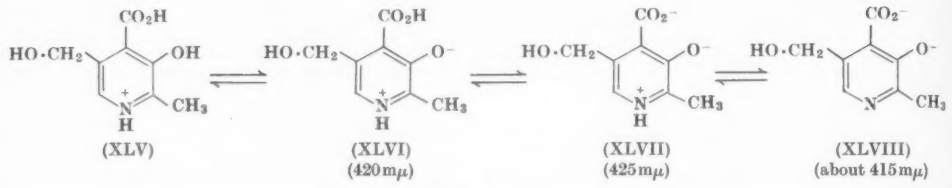
Fig. 7. Variation of (*a*) fluorescence intensity and of (*b*) fluorescence (Fl.) and excitation (Exc.) wavelengths with pH and H_0 of pyridoxic acid (●—●) and pyridoxic acid lactone (×—×) (concentration of each 5μg./ml.). H_0 is the acidity function of Hammett (see the text). Intensity values are arbitrary, that of the 3-hydroxypyridine anion at pH 11 being taken as 100.

excitation wavelengths that would be normally expected.

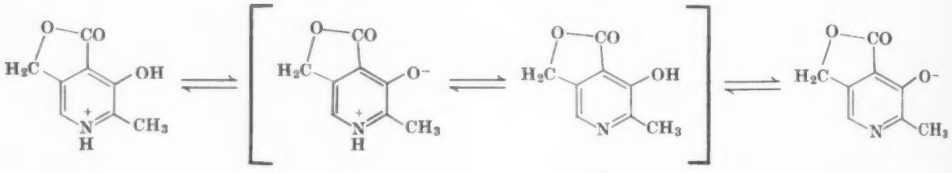
No fluorimetric evidence was found for the existence of the free aldehyde form of pyridoxal 5-phosphate below pH12. At low pH values the excitation wavelength 302mμ corresponds to the absorption maximum of the hydrated form of the cation of pyridoxal 5-phosphate, i.e. 291mμ, and not to that of the free aldehyde form, i.e. 342mμ (see Metzler & Snell, 1955). However, at high pH values, i.e. 12–14, the solution turns yellow and exhibits two excitation maxima and two corresponding fluorescence maxima. One is recorded in Fig. 6, i.e. $\lambda_{fl.}$ 370mμ and $\lambda_{exc.}$ 315mμ. The other, which was considerably weaker, occurred at $\lambda_{fl.}$ 525mμ and $\lambda_{exc.}$ 400mμ. This may be due to the trianion of the free aldehyde form (XLIV), since Williams & Neilands (1954) have recorded an absorption peak at 390mμ for pyridoxal phosphate in alkaline solution that may be due to the free aldehyde form (Metzler & Snell, 1955).

Pyridoxic acid and its lactone. The changes in fluorescence intensity and in excitation and fluorescence wavelengths with pH of pyridoxic acid and its lactone are shown in Fig. 7 (see also Table 4). In strongly acidic solutions (H_0 0 to −8·5, i.e. 1·08–34·5N-sulphuric acid) the fluorescence characteristics of the acid and lactone are similar, but above pH0 they differ considerably. The fluorescent forms of pyridoxic acid that could be expected to occur in solution are the cation (XLV), dipolar ion (XLVI), tripolar ion (XLVII) and dianion (XLVIII) (other forms can occur but they are disregarded to avoid complicating the picture). Below pH0, the changes are difficult to explain (see below). The dipolar ion (XLVI) appears to fluoresce intensely at 420mμ ($\lambda_{exc.}$ 320mμ), its maximum intensity occurring at pH2–4. This intensity is nearly 8 times that of the 3-hydroxypyridine anion. The pK_a of the ionization of (XLV) into (XLVI), estimated from the intensity curve, is 0·4. At pH5 both excitation and fluorescence wavelengths and fluorescence intensity change slightly with the ionization of the carboxyl group, i.e. with the conversion of (XLVI) into (XLVII). The pK_a values estimated from the intensity, excitation and fluorescence curves are 4·8, 5·25 and 5·25 respectively [Huff & Pealzweig (1944) give pK_a 5·5]. At about pH9 the intensity falls, but there is no change in excitation or fluorescence wavelength until pH12 is reached. This fall in intensity is interpreted as being due to the formation of the weakly fluorescent dianion (XLVIII) by deprotonation of the ring nitrogen atom. From the change in the intensity curve, it can be estimated that the pK_a for the conversion of (XLVII) into (XLVIII) is about 10·8. The weak fluorescence of the dianion (XLVIII) is probably due to the combined ring-deactivating effect of the uncharged ring nitrogen atom and the CO_2^- group.

Pyridoxic acid lactone (see Fig. 7) shows a completely different picture from the acid, since its main fluorescence occurs at pH8–13 at 430mμ ($\lambda_{exc.}$ 360mμ). This fluorescence is very intense, being 20 times that of the 3-hydroxypyridine anion. The forms of the lactone that might be expected are the cation (XLIX), dipolar ion (L), neutral form (LI) and anion (LII). The dipolar ion (L) is



(XLV) (XLVI) (420mμ) (XLVII) (425mμ) (XLVIII) (about 415mμ)



(XLIX) (L) (423mμ) (LI) (350mμ in ethanol) (LII) (430mμ)

very weakly fluorescent compared with the corresponding dipolar ion (XLVI) of the acid. The dipolar ion (L) fluoresces at 423mμ ($\lambda_{exc.}$ 365mμ) with an intensity of 35 compared with 770 for the dipolar ion (see Table 4). Here the formation of the lactone ring appears to have suppressed fluorescence intensity, a fact that is not easy to explain in view of the fluorescence of the anion (LII). The pK_a of the conversion of (XLIX) into (L), estimated from the excitation curve, is 1·6. At pH 5, the dipolar ion (L) ionizes to the intensely fluorescent anion (LII), the pK_a of the change, estimated from the fluorescence-intensity curve, being 6·95. Pyridoxic acid lactone is a derivative of the aza-isobenzofuran, furo[4,3-*c*]pyridine. The isobenzofurans (cf. LIV) are highly fluorescent compounds, presumably because of their ability to form free radicals (see Adams & Gold, 1940), and it is possible that an aza derivative such as the anion of pyridoxic acid lactone (LIII) is fluorescent for similar reasons. Several resonance structures for (LIII) which could contribute to its fluorescence can be drawn.

The fluorescent-wavelength curves suggest that three fluorescent forms of the acid and lactone occur below H_0 or pH 0. These forms (for the lactone) have fluorescent wavelengths of approx. 412, 451 and 423mμ, the excitation wavelength being 320mμ in each case. In 34·5N-sulphuric acid both pyridoxic acid and its lactone are strongly fluorescent at 412mμ (see Fig. 7). This may be due to a hydrogen-bonded cation of the acid or lactone (LV), which undergoes excited-state ionization to a form fluorescing at 451mμ with a much lower intensity and in which the hydrogen bond is ruptured. This second form then ionizes to the dipolar ion (L). Hydrogen-bonding is a factor that could tend to increase fluorescence (see Lippert, 1957).

Excited-state pK_a. Förster (1950) and Weller (1952) showed that the excitation by ultraviolet light of certain weak acids, such as α- and β-naphthol, enhanced their protolytic dissociation, with the result that the dissociation constants of the acids in the excited state (pK^*_a) were greater than those in the ground state (pK_a) by a factor 10^6–10^7. Weller (1952) derived a relationship between pK_a and pK^*_a from the Förster cycle (see Jaffé, Beveridge & Jones, 1964) for absorption and fluorescence, which is:

$$pK_a - pK^*_a = \frac{\Delta E - \Delta E'}{2{\cdot}303\boldsymbol{R}T} \qquad (1)$$

where ΔE and $\Delta E'$ are the transition energies from the ground state to the first excited singlet-state level of the acid and anion (or conjugate base) respectively, $\boldsymbol{R}$ is the gas constant and T is the absolute temperature. If $\boldsymbol{R}$ is replaced by $\boldsymbol{k}$, the Boltzmann constant, the equation then applies to one molecule.

Energy (E) is related to light frequency (ν) by the relation:

$$E = \boldsymbol{h}\nu$$

($\boldsymbol{h}$ is Planck's constant), and, since $\nu = c/\lambda$ (c is the velocity of light and λ is the wavelength in cm.), then:

$$E = \boldsymbol{h}c/\lambda$$

Substituting for ΔE and $\Delta E'$ in eq. (1) and expressing λ in mμ, we get:

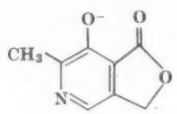
$$pK_a - pK^*_a = \frac{10^7\boldsymbol{h}c}{2{\cdot}303\boldsymbol{k}T}\left(\frac{\lambda' - \lambda}{\lambda\lambda'}\right) \qquad (2)$$

Table 4. *Fluorescence of pyridoxic acid and its lactone*

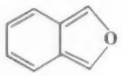
The concentration of each compound was 5 μg./ml.

Compound	Molecular species	Wavelength (mμ)			Relative fluorescence intensity†		pH range of maximum fluorescence	pK_a			
		Of max. absorption	Of max. excitation	Of max. fluorescence	Observed	Corrected		Int.	Exc.	Fl.	Literature values
Pyridoxic acid lactone	Cation 1*	315	320	about 412	about 800	820	H_0 − 8·8	−8·0	—	−7·9	
	Cation 2*	315	320	451	28·5	32	H_0 − 7·16 to −3·7	—	—	−2·1	
	Cation 2 (excited state)*	315	320	423	15	16	H_0 − 0·1 to pH2	—	—	1·6	
	Dipolar ion	357	365	423	32	19	pH2·5–4·8	—	—	6·95	7·05‡
	Anion	350	360	430	2150	1430	pH8·7–13				
Pyridoxic acid	Cation 1*	317	320	412	about 600	615	H_0 − 8·8	−7·9	—	−7·8	
	Cation 2*	316	320	446	38·5	42	H_0 − 7·1 to −3·7	—	—	−2·0	
	Cation 2 (excited state)*	316	320	420	36	39	H_0 − 0·1				
	Dipolar ion	316	320	420	770	810	pH1·5–4·0	0·5	—	—	
	Tripolar ion	307	315	425	650	690	pH6·1–9·4	4·85	5·2	6·0	5·5‡
	Dianion	306·5	315	about 415	23	27	pH12–13	10·85	—	—	9·75‡

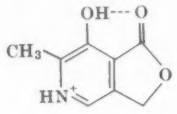
* See the text.
† See the text and Table 1.
‡ Huff & Pealzweig (1944).



(LIII)



(LIV)



(LV)

Table 5. *Excited-state dissociation constants of the cations of pyridoxal and related compounds*

Compound	pK^*_A of cation: Observed*	pK^*_A of cation: Calc.†	pK_A‡	pK_1‡	$pK_A - pK^*_A$ (obs.)
3-Hydroxypyridine	−3·0	−2·24	5·37§	5·10§	8·37
3-Hydroxy-*N*-methylpyridine	−3·0	−2·39	4·96‖	4·96‖	7·96
Pyridoxol	−2·2	−2·36	5·05§	5·00§	7·25
Pyridoxal (hemiacetal)	−3·3	−2·53	4·20§	4·20§	7·50
Pyridoxamine	−4·1	−4·25	3·32§	3·31§	7·42
Pyridoxal 5-phosphate (aldehyde hydrate)	< −2·5	−3·28	?	4·41¶	> 6·64
2-Hydroxypyridine	−3·3	−2·52	0·75‖	0·75‖	4·05
2-Hydroxy-*N*-methylpyridine	about −4·0	−3·48	0·32‖	0·32‖	4·32

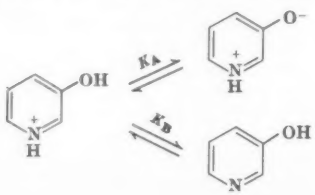
* Estimated from fluorescence data.
† Calculated by Weller's (1952) equation from pK_A values except for pyridoxal 5-phosphate.
‡ See the text.
§ Metzler & Snell (1955).
‖ Mason (1959).
¶ Williams & Neilands (1954).

where λ and λ' are the maximum absorption or fluorescence wavelengths of the acid and anion (or conjugate base) respectively. Substituting the known values for the constants h, c and k, and taking T as 293°A (20°C), eq. (2) becomes:

$$pK_a - pK^*_a = 21187\left(\frac{\lambda'-\lambda}{\lambda\lambda'}\right) \qquad (3)$$

Absorption wavelengths are more accurately measured than fluorescence wavelengths on present-day instruments, and can be determined with an accuracy of 1mμ. Therefore in Table 5 λ and λ' used in calculating pK_a^* are absorption wavelengths.

When the 3-hydroxypyridine cation ionizes, it can lose a proton from the hydroxyl group or the positively charged nitrogen atom thus:



The overall dissociation constant is pK_1, and pK_A and pK_B are the constants for the ionizations shown above. These constants are related, for:

$$K_1 = K_A + K_B$$

The excited pK determined fluorimetrically is pK^*_A, since the neutral molecule is virtually non-fluorescent. pK^*_A can also be calculated by Weller's (1952) equation above from published values of pK_A. For most of the compounds given in Table 5, pK_1 and pK_A are almost identical because, in these cases, very little of the neutral molecule occurs in aqueous solution (Metzler & Snell, 1955). Table 5 gives pK^*_A values estimated from our fluorescence data compared with those calculated from Weller's (1952) equation. The observed values are of the right order and the agreement justifies the use of the acidity function, H_0, for very strongly acidic solutions. The values for $(pK_A - pK^*_A)$ are all about 7–8 for the 3-hydroxypyridine compounds and this indicates that the behaviour of the cations is similar. The value for both the 2-hydroxypyridines is about 4.

Stokes shifts. Stokes (1852) showed qualitatively that the wavelength of fluorescence is longer than that of the corresponding excitation. The differ-

Table 6. *Stokes shifts for pyridoxol and related compounds*

Stokes shift in cm.$^{-1}$ = 10^7 $(1/\lambda_{exc.} - 1/\lambda_{fl.})$, λ being in mμ. — means either that the form does not exist or that it is non-fluorescent.

	Stokes shift (cm.$^{-1}$)				
Compound	Cation	Cation ionizing in excited state	Dipolar ion	Neutral form†	Anion
3-Hydroxypyridine	5750	9830	6240	4520	6160
3-Methoxypyridine	4150	—	—	—	—
3-Hydroxy-*N*-methylpyridine	4690	9400	5910	—	—
Pyridoxol	5210	9420	4920	5430	5870
Pyridoxal	5980	8470	5300	5220	5560
Pyridoxamine	5510	9310	5730	—	5090
Pyridoxal 5-phosphate	*	9360	5860	5500	5760
Pyridoxic acid	7700	9530	8150	—	8370
Pyridoxic acid lactone	7700	9770	3870	—	4140
2-Hydroxypyridine	7110	8320	6230	6300	5870
2-Methoxypyridine	4880	—	—	5090	—
2-Hydroxy-*N*-methylpyridine	6850	8440	6160	—	—

* The fluorescence wavelength could not be measured.
† In ethanol.

ence between the wave number of the longest wavelength of the excitation maximum and that of the shortest wavelength of the fluorescence maximum is referred to as the 'Stokes shift' (Leemann, Stich & Thomas, 1963) and is a measure of the energy involved when a compound is raised to the excited state. It is also of structural significance (see Leemann *et al.* 1963). The Stokes shift, in cm.$^{-1}$, can be calculated from the expression:

$$\text{Stokes shift} = 10^7(1/\lambda_{exc.} - 1/\lambda_{fl.})$$

where $\lambda_{exc.}$ and $\lambda_{fl.}$ are the maximum excitation and fluorescence wavelengths expressed in mμ.

The Stokes shifts for the various species of the compounds studied in this paper are given in Table 6. The first nine compounds are derivatives of 3-hydroxypyridine, and, apart from pyridoxic acid and its lactone, the values in each column are similar. The Stokes shift for the cations, dipolar ions, neutral forms and anions of the first seven compounds is 4000–6000 cm.$^{-1}$, whereas the value for the cation undergoing excited-state ionization is 8000–10000 cm.$^{-1}$. For these seven compounds the similarity of the values in each column supports the view that the ionic species considered are similar, and supports our interpretation of the fluorescence data. The larger values for the cations undergoing excited-state ionization indicate that a process in addition to absorption and emission is involved. In fact, some of the absorbed energy is being used to bring about ionization in the excited state. The values for the cation, dipolar ion and anion of pyridoxic acid and its lactone do not agree with the others or with one another except for the cations, and the interpretation of the fluorescence data may be more complicated than has been suggested.

The 2-hydroxypyridines give another series of Stokes shifts. The values for the dipolar ion and anion are similar to those of 3-hydroxypyridine and suggest similarity between these forms of the two isomers. Again, 2-methoxypyridine, which cannot occur as a pyridone, falls in line with the 3-methoxypyridine. The 2-hydroxypyridine cation and neutral form and 2-hydroxy-*N*-methylpyridine cation have Stokes shifts that are larger than those of the 3-hydroxypyridines, and this may be due to their ability to exist as pyridones. The Stokes shifts have not been extensively studied, but their possible uses have been discussed by Leemann *et al.* (1963).

This work was supported by a grant from Dr Salsbury's Laboratories, Charles City, Iowa, U.S.A. We are grateful to the American Instrument Co., Silver Spring, Md., U.S.A., for gifts of equipment.

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The Biohydrogenation of α-Linolenic Acid and Oleic Acid by Rumen Micro-organisms

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(*Received* 1 *July* 1965)

1. α-[U-^{14}C]Linolenic acid was incubated with the rumen contents of sheep and the metabolic products were characterized by thin-layer chromatography, gas–liquid chromatography and absorption spectroscopy in the ultraviolet and infrared. 2. A tentative scheme for the biohydrogenation route to stearic acid is presented. The main pathway is through diconjugated *cis–cis–cis*-octadecatrienoic acid, non-conjugated *trans–cis* (*cis–trans*)-octadecadienoic acid and *trans*-octadecenoic acid, but other pathways are apparent. 3. Washed rumen micro-organisms possessed only a limited capacity to hydrogenate α-linolenic acid and oleic acid but the rate was greatly stimulated by a factor(s) present in the supernatant rumen liquor. 4. Pure cultures of *Clostridium perfringens*, *Streptococcus faecalis*, *Escherichia coli* and a coliform organism isolated from sheep faeces possessed negligible ability to hydrogenate unsaturated fatty acids compared with a mixed population of rumen micro-organisms. *Butyrivibrio fibrisolvens* slowly converted linoleic acid into octadecenoic acid.

Biohydrogenation of unsaturated fatty acids is a characteristic reaction carried out by micro-organisms of the rumen (Shorland, Weenink, Johns & McDonald, 1957; Garton, 1964; Ward, Scott & Dawson, 1964) and lower digestive tract (Ward *et al.* 1964). The main fatty acid in the natural pasture diet of the ruminant is *cis–cis–cis*-$\Delta^{9,12,15}$-octadecatrienoic acid (α-linolenic acid), which is esterified in a number of complex lipids; esterified linoleic acid and oleic acid are also present. Although it is possible that some of these esterified unsaturated fatty acids may be hydrogenated directly, it is likely that most are enzymically hydrolysed to the free acid (Garton, 1964) before hydrogenation takes place.

When linolenic acid is added to an artificial rumen it is hydrogenated to stearic acid via dienoic and monoenoic acid intermediates with no appreciable conversion into other metabolic products (Ward *et al.* 1964). Wood, Bell, Grainger & Teekell (1963) introduced radioactive linoleic acid into the intact rumen and later isolated most of the radioactivity from the rumen contents as C_{18} monoenoic acid and stearic acid.

In the present paper a study has been made of the initial metabolic products of the hydrogenation of α-linolenic acid and their place in the hydrogenation pathway. In addition, the capacity of whole and disrupted washed rumen micro-organisms to hydrogenate linolenic acid and oleic acid has been examined. The clear rumen-liquor supernatant has been shown to play an important role in the hydrogenation sequence.

EXPERIMENTAL

Rumen samples. Rumen contents for experiments *in vitro* were withdrawn from Clun Forest sheep with rumen fistulae fed on a hay–oats diet (5:1, w/w; 1200g./day). The fluid was strained through four layers of cotton gauze to remove coarse food particles.

Cultures of pure bacteria. The following bacteria were grown in pure culture: *Butyrivibrio fibrisolvens* (Dr P. N. Hobson, Rowett Research Station, Aberdeen); *Escherichia coli* B and *Streptococcus faecalis* Dunn (Dr G. S. Coleman, Institute of Animal Physiology, Babraham, Cambridge); a coliform organism (a motile lactose-fermenter) isolated from sheep faeces by Mr R. W. White; *Clostridium perfringens* S 107 (Wellcome Research Laboratories, Beckenham, Kent). The organisms were grown anaerobically in 0·5% yeast–1% peptone broth (*E. coli* and *S. faecalis*), fortified casein hydrolysate (*C. perfringens*) or reinforced clostridial medium (*B. fibrisolvens*) and tested for their ability to hydrogenate unsaturated fatty acids towards the end of the exponential phase of growth.

Washed rumen micro-organisms. The strained rumen contents were centrifuged at 23500*g* for 30min. in a Servall centrifuge at 4°. The supernatant (rumen liquor) was decanted and the sediment suspended in the same volume of a reducing buffered saline, pH 7·0, prepared as

follows: NaCl (8·5g.) and 4·1ml. of conc. HCl were added to 1l. of water and the solution was boiled. After cooling, 6·06g. of tris and 200mg. of cysteine hydrochloride in 1·2ml. of N-NaOH were added and the whole was covered with a layer of liquid paraffin. The suspension was centrifuged as before and the sediment rewashed a further two times. The washed micro-organisms were suspended in the original volume of buffered saline for incubation.

Disruption of micro-organisms. Washed micro-organisms were disintegrated either by 20min. irradiation at 0° with ultrasound (MSE 60w disintegrator) or in a Mickle shaker for 30min. at 4° with 20% (by vol.) of Ballotini no. 13 glass beads and no air space.

Microbial incubation with radioactive fatty acid. The radioactive fatty acid was dissolved in 0·05–0·1ml. of methanol in a 50ml. pear-shaped flask and an equal volume of buffered saline was added. The emulsion was evaporated under vacuum to remove most of the methanol and 2–10ml. of the rumen preparations was added. Incubation was carried out at 38° with continuous gassing with water-saturated N_2+CO_2 (95:5) to stir and maintain anaerobic conditions in and above the preparation.

Extraction of fatty acids from the incubation mixture. The incubation mixture (10ml.) was shaken with 100ml. of chloroform–methanol–conc. HCl (50:50:1, by vol.) containing 2,6-di-*tert.*-butyl-4-methylphenol (20mg.) as an antioxidant. After standing 10min. the suspension was filtered and the filtrate shaken with 20ml. of water; the lower layer was removed and evaporated to dryness on a rotary evaporator. The residue was extracted with 20ml. of ether, the ethereal solution evaporated to dryness and 5ml. of 6% (w/v) KOH in ethanol–water (19:1, v/v) added. After refluxing for 1hr., 5ml. of water was added and the mixture was extracted three times with equal volumes of light petroleum (b.p. 40–60°). The aqueous phase was acidified with 5N-HCl and extracted twice with equal volumes of light petroleum (b.p. 40–60°). The light-petroleum extracts, containing the fatty acids, were evaporated to dryness.

Gas–liquid chromatography. Gas–liquid chromatography was carried out as described by Ward *et al.* (1964) and collection of samples from preparative columns by the procedures of Scott, Ward & Dawson (1964). Radioactive scanning of the gas–liquid-chromatogram effluent was performed with an apparatus similar to that of James & Piper (1961) and whose construction was described by Scott *et al.* (1964). Fatty acids were methylated with diazomethane in methanol–ether (1:9, v/v) (Schlenk & Gellerman, 1960).

Thin-layer chromatography. The methyl esters of the geometric isomers of C_{18} mono-, di- and tri-enoic acids were separated on thin-layer plates of silica gel G (E. Merck A.-G., Darmstadt, Germany) impregnated with 5% (w/w) $AgNO_3$ (de Vries, 1962, 1963; Ward *et al.* 1964). Benzene–hexane (1:1, v/v) was used to separate monoenoic acid and stearic acid, and benzene–ether (99:1, v/v) was used to examine the dienoic acids. The fatty acid methyl esters were detected either by spraying with 0·2% (w/v) 2,7-dichlorofluorescein in ethanol and examination under u.v. light or by spraying with aq. 50% (v/v) H_2SO_4 and then heating at 100°. Radioactive spots were located either by scanning with a mica-window Geiger–Müller counter (Ward *et al.* 1964) or by radioautography with Kodak 0·800 glass photographic plates. If the methyl esters were not being recovered, the silica gel was sprayed with Neatan varnish (Merck) before radioautography to facilitate handling. For recovery of the methyl ester spots, the silica gel corresponding to the radioactive area was sucked up into a small sintered-glass column (Ward *et al.* 1964) and then eluted with ether.

Oxidation of fatty acids. It was found that the procedure of Rudloff (1956) for oxidation at the double bond, though satisfactory for monoenoic acid, gave extensive over-oxidation with dienoic and trienoic acids. Consequently these were oxidized by the alkaline-permanganate method of Lapworth & Mottram (1925) suitably scaled down. This gave much better yields of the expected di- and mono-carboxylic acids from known linolenic acid and linoleic acid samples. After oxidation the acidified solutions were exhaustively extracted with light petroleum, and the oxidation products were methylated and examined by gas–liquid chromatography on a polyethylene glycol adipate column isothermally at 150°. Complete separation of the mono- and di-carboxylic acids was obtained.

Radioactive counting and materials. Methods were as described by Ward *et al.* (1964). The all-*cis*-[U-^{14}C]- and -[1-^{14}C]-fatty acids were obtained from The Radiochemical Centre, Amersham, Bucks. [1-^{14}C]Elaidic acid was obtained by the Se-catalysed isomerization of [1-^{14}C]oleic acid (Swern & Scanlan, 1953). The α-[U-^{14}C]linolenic acid showed no radioactive impurities on gas–liquid chromatography on a polyethylene glycol adipate column with radioactive scanning. Thin-layer chromatography on silica gel–5% $AgNO_3$ plates also failed to show any radioactive component other than α-linolenic acid.

RESULTS

Biohydrogenation of α-linolenic acid by whole rumen contents. Rumen contents were incubated with α-[U-^{14}C]linolenic acid under anaerobic conditions, and thin-layer chromatograms on silica gel–silver nitrate plates were prepared of the fatty acids isolated from the mixture. Radioautographs showed that in 2hr. the α-linolenic acid had been substantially converted into stearic acid, although some C_{18} *trans*-monoenoic acid was also present. With shorter periods of incubation radioautography revealed a complex pattern of radioactive degradation products (Fig. 1) with the transient accumulation of C_{18} dienoic acid intermediates that had been observed by Ward *et al.* (1964). No hydrogenation occurred when the α-linolenic acid was incubated under similar conditions with 0·9% sodium chloride in place of rumen contents. When incubations were carried out for different time-intervals it became apparent from radioautography of the thin-layer plates that there were well-defined sequential changes in the metabolic products formed (Fig. 2). By choosing suitable incubation times it was possible to obtain a sufficient accumulation of the radioactive intermediates to isolate them by preparative thin-layer chromatography. This allowed their gas–liquid chromatographic properties to be investigated: the radioactivity in the effluent from the columns was continually

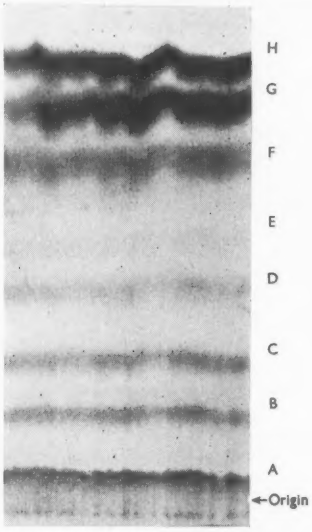


Fig. 1. Thin-layer chromatogram of the fatty acids produced on incubation of α-[U-^{14}C]linolenic acid with rumen contents. A 0·5mg. sample (6μc) of α-[U-^{14}C]linolenic acid was incubated at 37° for 30min. with 4ml. of strained rumen contents. The fatty acids were isolated and after methylation run as a strip on a thin-layer-chromatographic plate in benzene–ether (99:1, v/v). Radioautography was carried out for 18hr. The trace radioactive component E is not usually seen.

Table 1. *Gas–liquid chromatography of methyl esters of fatty acids formed from α-linolenic acid*

Spots on thin-layer chromatograms were located by radioautography. Gas–liquid-chromatographic peaks were located by continuous combustion of the gas effluent and detection of the $^{14}CO_2$ in a proportional counter.

Experiment	Spots of thin-layer chromatogram (Fig. 1) with R_F	Retention volume relative to methyl palmitate: On 10% Apiezon L (200°)	Retention volume relative to methyl palmitate: On 10% polyethylene glycol adipate (180°)
Whole rumen contents	A (0·15)	1·88	3·23
	B (0·30)	a 2·31	4·39
		b 1·87	2·67
		c 1·95	3·34
	C (0·39)	a 1·94	2·67
	D (0·56)	a 1·99	2·55
	F (0·70)	a 2·21	2·37
		b 1·97	2·11
	G (0·82)	2·07	2·14
	H (0·91)	2·28	1·97
Washed rumen contents	B	a 2·37	4·28
		c 2·02	3·29
		d 1·92	5·10
	C	a 2·05	2·56
		b 2·93	5·15
	D	a 1·90	2·56
		b 2·99	3·25
		c 2·55	4·62

scanned in a proportional counter after oxidation of any emerging radioactive fatty acid to $^{14}CO_2$. It became obvious that some of the apparent constituents that ran as single spots on thin-layer chromatography were resolved into two or more components on gas–liquid chromatography (Table 1). For clarity these components have been labelled according to: (1) their running on thin-layer plates of silica gel–silver nitrate; (2) their resolution on gas–liquid chromatography (see Fig. 1 and Table 1). By using large-scale incubations of rumen contents (200ml.) with α-[U-^{14}C]linolenic acid plus 10mg. of unlabelled α-linolenic acid it was possible to isolate sufficient quantities of some of the intermediates to examine their infrared and ultraviolet spectra.

Identity of radioactive components formed on incubating α-linolenic acid with whole rumen contents. Component A. Gas–liquid chromatography (Table 1) indicated that this was the original α-linolenic acid. Zero-time incubations (Fig. 2) showed that it contained little contaminating material. The radioactive material remaining at the zero-time origin (Fig. 2) was probably autoxidized fatty acid (see Nichols & Morris, 1964).

Component Ba. Catalytic hydrogenation (Farquhar, Insull, Rosen, Stoffel & Ahrens, 1959) yielded stearic acid as the sole product. The fatty acid possessed a strong absorption peak in ethanol at 233mμ, which is characteristic of a conjugated dienoic acid (Holman, 1957). However, the gas–liquid-chromatographic (Table 1) and thin-layer-chromatographic (Fig. 1) properties were different from the expected behaviour of a conjugated octadecadienoic acid (Ward *et al.* 1964; de Vries, 1962, 1963). Alkaline isomerization (Holman, 1957) produced a main absorption peak at 268mμ, characteristic of a triconjugated trienoic acid. This indicated that the fatty acid is a trienoic acid in which two of the double bonds are conjugated. Oxidation of the acid gave a gas–liquid-chromatographic picture that was very similar to that from α-linolenic acid, i.e. propionic acid and azelaic acid were the main products. The fatty acid is therefore probably $\Delta^{9,11,15}$- or $\Delta^{9,13,15}$-octadecatrienoic acid. In the

infrared no distinct *trans* peak was visible although a weak peak at 978 cm.$^{-1}$ was apparent, so that the fatty acid probably has a *cis–cis–cis* configuration.

Component Bb. This fatty acid occurred as a minor transient intermediate in the biohydrogenation process. Thin-layer chromatography with light petroleum–ether (99:1, v/v) solvent did not separate it appreciably from component Ba but a slightly better differentiation was apparent in benzene–ether (9:1, v/v). Its gas–liquid-chromatographic retention volumes and its R_F on thin-layer chromatography indicated that the acid is a *cis–cis*-octadecadienoic acid. In a previous investigation this was noted as an intermediate in the ruminal hydrogenation of linolenic acid (Ward *et al.* 1964).

Component Bc. This fatty acid was never more than a very minor component of the incubation medium and was not always found. Its gas–liquid-chromatographic retention volumes and behaviour on thin-layer chromatography suggest that it is a geometric isomer of non-conjugated octadecatrienoic acid. As small amounts of a fatty acid of identical chromatographic properties were formed when α-linolenic acid was incubated with 0·9% sodium chloride at 37° under anaerobic conditions, it is probably not a true intermediate in the metabolic pathway of α-linolenic acid hydrogenation in the rumen.

Component Ca. The gas–liquid-chromatographic retention volumes of this acid (Table 1) indicate that it is a non-conjugated octadecadienoic acid. Its infrared spectrum in carbon disulphide showed a strong peak at 969 cm.$^{-1}$, indicative of the *trans* configuration. This and its behaviour on thin-layer chromatography indicates that it is a non-conjugated *cis–trans* (*trans–cis*)-octadecadienoic acid.

Component Da. The gas–liquid-chromatographic retention volumes again indicate that, like component Ca, this is a non-conjugated octadecadienoic acid. The infrared spectrum in carbon disulphide showed a *trans* peak at 969 cm.$^{-1}$. From its behaviour on thin-layer chromatography a *trans–trans*-octadecadienoic acid is indicated, or a positional isomer of component Ca [i.e. a non-conjugated *cis–trans* (*trans–cis*)-octadecadienoic acid]. From the ready interconversion of components Da and Ca and the intensity of the *trans* peak the latter identification is more likely.

Component Fa. The gas–liquid-chromatographic retention volumes did not correspond to any recorded C_{18} fatty acid and not enough was present to isolate sufficient for studies of its absorption spectra. Its specific radioactivity was extremely high compared with the other fatty acid intermediates and this suggests that it may not be a normal product of α-linolenic acid hydrogenation in the rumen.

Component Fb. The gas–liquid-chromatographic retention volumes and thin-layer-chromatographic properties are consistent with this being *cis*-octadecenoic acid.

Component G. The gas–liquid-chromatographic properties indicate this is a *trans*-octadecenoic acid. In previous studies this was identified as an intermediate of the ruminal metabolism of α-linolenic acid (Ward *et al.* 1964).

Component H. This fatty acid behaves identically with stearic acid on gas–liquid and thin-layer chromatography.

Sequence of metabolic changes during hydrogenation of α-linolenic acid. Thin-layer chromatography (Fig. 2) and gas–liquid chromatography of serial samples from an incubation mixture indicated that the *cis–cis–cis*-octadecatrienoic acid with two of the double bonds conjugated (component Ba) was the initial product of α-linolenic acid metabolism in the rumen. This was followed by hydrogenation at the conjugated double bond, this process being associated with a considerable *cis–trans* isomerization of the remaining double

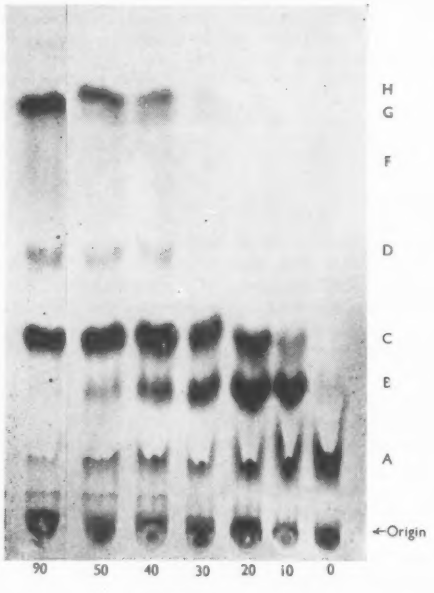


Fig. 2. Incubation of α-[U-^{14}C]linolenic acid with rumen contents for various time-intervals. Experimental details are given in Fig. 1 and the Experimental section.

bond. The main products were *cis–trans* (*trans–cis*)-octadecadienoic acid (component Ca) and to a smaller extent *cis–cis*-octadecadienoic acid (component Bb) (Fig. 2). Also, an isolated B fraction from a thin-layer chromatogram gave predominantly *cis–trans* (*trans–cis*)-octadecadienoic acid (fraction C) on incubation for 30 min. with rumen contents (Fig. 3). The *cis–trans* (*trans–cis*)-octadecadienoic acid (component Ca) could be slowly isomerized to the minor unknown octadecadienoic acid (component Da) (Figs. 2 and 3), the reaction being reversible (Fig. 3). The *cis–trans* (*trans–cis*)-octadecadienoic acid was then hydrogenated, giving mainly *trans*-octadecenoic acid (Figs. 2 and 3) and probably a small amount of the *cis* isomer (see Ward *et al.* 1964). The C_{18} monoenoic acids were then rapidly hydrogenated to stearic acid.

Biohydrogenation of α-linolenic acid by washed rumen micro-organisms. On centrifuging rumen contents and examining the micro-organisms and supernatant separately, it became clear that the capacity to hydrogenate was solely confined to the micro-organisms. After the complete removal of the supernatant liquor from the micro-organisms, incubation with α-[U-^{14}C]linolenic acid for 2 hr. resulted in the slow accumulation of metabolic products, which, on thin-layer chromatography on silica gel–silver nitrate plates, exactly corresponded to spots B, C and D (see Fig. 1).

However, in this case, in spite of the long period of incubation, only limited amounts of monoenoic acid and stearic acid had been formed. In addition, on isolation of the spots from the thin-layer chromatograms and examination by gas–liquid chromatography it became apparent (Table 1) that, though fraction B contained the products formed by whole rumen contents, fractions C and D were far more complex and somewhat variable in composition.

When the mixture of fatty acids formed by incubating washed rumen micro-organisms with radioactive α-linolenic acid were isolated and incubated with rumen-liquor supernatant, there was no conversion into monoenoic acid or stearic acid. However, the addition of the supernatant liquor to the washed rumen micro-organisms completely restored their original hydrogenation capacity towards α-linolenic acid. Clearly therefore rumen liquor contains some factor that assists the micro-organisms to hydrogenate α-linolenic acid completely.

This essential factor(s) in rumen liquor was only partially destroyed by boiling for 5 min. On treating the liquor with 9 vol. of ethanol, it was concentrated in the ethanol-insoluble fraction. It could not be replaced by $NADH_2$, $NADPH_2$ (1·4 μmoles/ml.), succinate, lactate or glucose.

Disruption of the washed rumen micro-organisms with ultrasound (15 min. in the MSE 60 w disintegrator) resulted in loss of ability to carry out hydrogenation, although some conversion into the diconjugated octadecatrienoic acid was observed.

Biohydrogenation of oleic acid. When [U-^{14}C]-oleic acid was incubated with rumen contents, radioautographs of the thin-layer chromatograms of the resultant fatty acids indicated rapid conversion into *trans*-monoenoic acid and stearic acid. Neither the whole washed micro-organisms nor homogenates of these produced on disintegration with ultrasound, butanol treatment or in the Mickle shaker could hydrogenate oleic acid or elaidic acid. By itself rumen liquor was incapable of hydrogenating oleic acid, but on the addition of this to washed rumen micro-organisms their ability to convert oleic acid into *trans*-monoenoic acid and stearic acid was fully restored. Boiling the rumen liquor for 5 min. partly destroyed this capacity and its action could not be replaced by $NADH_2$ and $NADPH_2$.

Ability of pure cultures of bacteria to carry out biohydrogenation of unsaturated fatty acids. A number of pure cultures of anaerobic or facultative anaerobic bacteria found in the intestinal tract

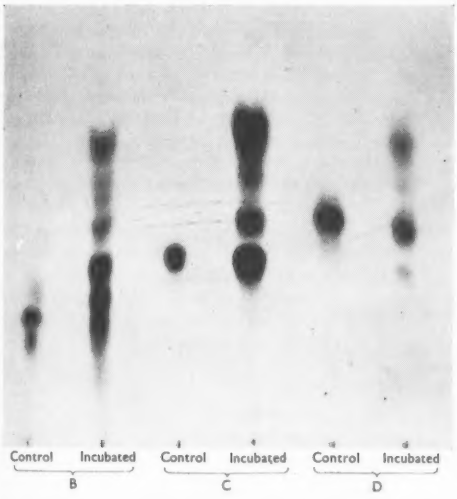


Fig. 3. Incubation of the isolated intermediates in α-linolenic acid hydrogenation with rumen contents. Intermediary fractions B, C and D were isolated from a 30 min. incubation of α-[U-^{14}C]linolenic acid with rumen contents by preparative thin-layer chromatography of the fatty acid methyl esters. After saponification the free fatty acid fractions obtained (B, C and D) were again incubated individually for 30 min. with rumen contents. The fatty acids were extracted, methylated and subjected to thin-layer chromatography and radioautography.

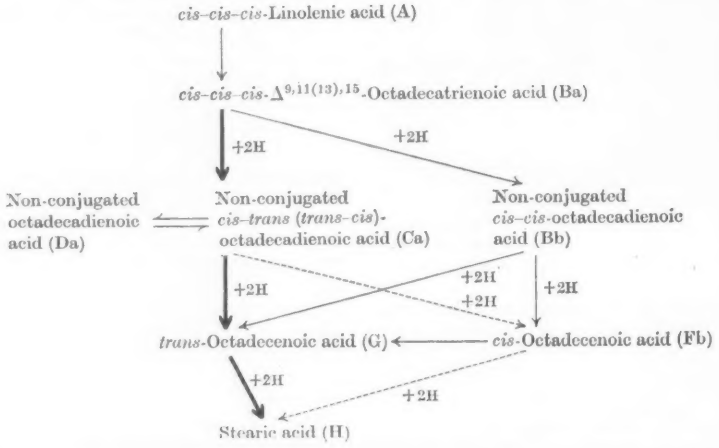
were tested to determine whether they possessed any ability to hydrogenate unsaturated fatty acids. These included *C. perfringens* S107, *S. faecalis* Dunn, *E. coli* B and *B. fibrisolvens*. The actively growing pure cultures were incubated anaerobically for 2–3 hr. with the radioactive fatty acid, and the fatty acids were extracted and examined by thin-layer chromatography as before. *S. faecalis* did not metabolize [U-^{14}C]linolenic acid. The *C. perfringens* culture completely metabolized added [1-^{14}C]linoleic acid but only a very little stearic acid was formed. The main product ran much slower than linoleic acid on thin-layer silica gel–silver nitrate plates and was possibly octadecatrienoic acid. Similarly, when [1-^{14}C]oleic acid was incubated with *C. perfringens* the main metabolic product was a dienoic acid or *trans*-trienoic acid, with only minimal amounts of stearic acid being formed (cf. Harris & James, 1965). When *E. coli* cultures were incubated with [U-^{14}C]linolenic acid a very slow conversion into stearic acid was observed. On recovering the organisms by centrifugation and washing them with reducing saline the ability to metabolize linolenic acid was lost. A coliform organism isolated from sheep faeces possessed no ability to hydrogenate α-[U-^{14}C]linolenic acid. *B. fibrisolvens* grown in the presence of linoleic acid for 21 hr. partially converted it into octadecenoic acid. However, when organisms in the exponential phase of growth were incubated for 2 hr. with linoleic acid under hydrogen, little hydrogenation of the fatty acid occurred.

DISCUSSION

Biohydrogenation of α-linolenic acid by rumen micro-organisms is clearly a very complex chemical process and in this respect is equivalent to its chemical hydrogenation catalysed by metals (Allen & Kiess, 1955; Scholfield *et al.* 1960).

Though certain fatty acids show a transient accumulation during the metabolic hydrogenation, it must be emphasized that these may not be the only intermediate products and in fact could be merely those whose further metabolism is a rate-limiting step. With this proviso in mind, the present results coupled with previous studies (Ward *et al.* 1964) allow a tentative metabolic scheme for the hydrogenation of α-linolenic acid to stearic acid to be formulated (Scheme 1). It must, however, be emphasized that this scheme will be additionally complicated by the considerable positional migration of the double bonds that can occur during hydrogenation (Shorland *et al.* 1957; Ward *et al.* 1964). The techniques used produce poor separation of the positional isomers of a given geometric fatty acid isomer and information about them is only likely to be obtained by a detailed study of the oxidation products.

It is clear from the present experiments that the full hydrogenation capacity of rumen micro-organisms towards α-linolenic acid and oleic acid requires an essential cofactor(s) that is present in the rumen-liquor supernatant. This is in agreement with the results of Polan, McNeill & Tove (1964), who reported that boiled rumen fluid stimulated



Scheme 1. Pathways of hydrogenation of α-linolenic acid by rumen micro-organisms. The letters in parentheses refer to the fractions described in Table 1 and in the text.

the hydrogenation of linoleic acid and oleic acid by rumen micro-organisms from a steer. The identity of this factor is unknown, but it is possible that it could be a substrate for a dehydrogenase that would supply reduced nicotinamide nucleotides. However, lactate, succinate and glucose were ineffective as substitutes.

Washed rumen micro-organisms on their own have only a limited capacity for hydrogenation, and fatty acids accumulate that are not normally found with whole rumen contents. Some of these may be conjugated. Shorland *et al.* (1957) observed the accumulation of conjugated dienoic acids when linoleic acid was incubated with rumen contents under non-physiological conditions, but none was formed from linolenic acid.

It is possible that these additional acids are on subsidiary pathways and only accumulate when the main pathway of hydrogenation is blocked by adverse physiological conditions, such as the absence of rumen liquor cofactor(s). Alternatively, they could be intermediates in the main hydrogenation pathway that are normally metabolized very quickly so that no appreciable concentration ever forms.

Our experiments with a limited number of pure cultures of bacteria typically found in the alimentary tract show that the ability to biohydrogenate rapidly linolenic acid to stearic acid is not possessed by all bacteria. A strain of *E. coli* showed some ability to hydrogenate linolenic acid to stearic acid, but the rate was very low compared with a mixed population of rumen micro-organisms. Polan *et al.* (1964) found that among 20 pure cultures of rumen bacteria only certain strains of *B. fibrisolvens* could hydrogenate linoleic acid, and then only to an octadecenoic acid, not stearic acid. We have confirmed this observation but in our hands the rate of hydrogenation was much lower and its extent more limited than that carried out by the mixed population of rumen micro-organisms. Presumably other species must exist in the rumen that are more efficient at hydrogenation and can convert the octadecenoic acid into stearic acid.

We are extremely grateful to Dr G. S. Coleman and Mr R. W. White, who prepared cultures of pure bacteria, and to Mr David Lander, who rendered valuable technical assistance. Dr P. N. Hobson and Dr G. S. Coleman are thanked for supplying slopes of various pure bacteria.

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Nutrition and Lysosomal Activity

THE INFLUENCE OF THE VITAMIN A STATUS ON THE PROTEOLYTIC ACTIVITY OF EXTRACTS FROM THE LIVERS AND KIDNEYS OF RATS

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(*Received* 31 *May* 1965)

1. Young rats were kept for several weeks on a diet deficient in vitamin A. Some were undosed, others were given marginal (25i.u. weekly), adequate (1000i.u. weekly) or excessive (50000i.u. daily) doses of vitamin A acetate. The undosed rats developed signs of vitamin A deficiency, and the overdosed animals had skeletal fractures indicative of hypervitaminosis A. 2. The rats were decapitated. Their livers, and sometimes their kidneys, were homogenized and processed by centrifugal methods to sediment most of the lysosome fractions. Proteolytic activity was measured, after treatment with a detergent, in the whole homogenate ('total' activity), in the pellet obtained after 20min. at 15000*g* ('bound' activity) and, without treatment with detergent, in the supernatant ('free' activity). 3. In rats suffering from hypervitaminosis A the free activity and to a smaller extent the total activity were increased. Free activity was also raised in most rats suffering from avitaminosis A, but less than in those suffering from hypervitaminosis. 4. The vitamin A status appeared to have little effect on the proteolytic activity of the kidneys. Results for total and free activities, but not for bound activities, were higher than for corresponding liver preparations. 5. Control experiments were done on starved rats and on rats which were pair-fed with hypervitaminotic animals. Short periods of starvation caused an increase in free activity in young rats, but not in adults. The increases caused by starvation were much less than those caused by hypervitaminosis A. 6. For studies of the distribution of vitamin A more complete separation of the subcellular fractions was carried out on the combined livers from several hypervitaminotic rats. The concentration of vitamin A in the lysosome fraction was less than in the liver as a whole. 7. Our finding that the free proteolytic activity of the liver is increased by toxic oral dosing with vitamin A can be considered an extension of the previous observation that proteolytic enzymes are liberated when lysosomes are treated *in vitro* with vitamin A.

According to de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) the acid proteases of cells together with a number of other acid hydrolases are concentrated in small organelles contained in the cytoplasm, to which particles the name 'lysosomes' is given. The treatment of isolated lysosomes *in vitro* with vitamin A causes the liberation of their proteolytic enzymes (Dingle, 1961) and other hydrolases (J. T. Dingle, unpublished work). This release of enzymes probably explains the loss of mucopolysaccharides from cartilage cultured in the presence of excess of vitamin A (3·0μg./ml.) (Dingle, Lucy & Fell, 1961) and its consequent loss of metachromasia (Fell & Mellanby, 1952). The distribution of vitamin A throughout the body, however, is well known to be highly localized, with the liver as the central storage depot and distribution centre. It is therefore always questionable how closely experiments *in vitro* can reproduce the exact conditions pertaining *in vivo*. In particular it might be asked whether ingested vitamin A can reach the lysosomes *in vivo* in concentrations equal to those used in experiments *in vitro*.

It seemed therefore desirable to ascertain whether the proteolytic activity of lysosomes is influenced by vitamin A reaching the cells *in vivo* by the normal routes of absorption and distribution. For this purpose homogenates and subcellular fractions have been made from the livers and kidneys of

groups of rats given graded intakes of vitamin A ranging from none to toxic quantities. A preliminary report of some of our findings (Dingle, Sharman & Moore, 1963) has already indicated that the proteolytic activity of tissue extracts can indeed be influenced by the dietary intake of vitamin A. In this paper our evidence is presented more fully. We have also studied the extent to which lysosomal activity can be directly related to the vitamin A status, rather than indirectly through a reduced food intake and consequent emaciation (Beaufay, van Campenhaut & de Duve, 1959).

EXPERIMENTAL

Preparation of rats with graded vitamin A status. Young piebald rats were obtained from mothers that had received a full stock diet during pregnancy, but from which green vegetables, liver and cod-liver oil were withdrawn when the offspring became 10 days old. The young continued on the same diet until the experiments started, usually when their body weights were about 70g. At this point measurements of the vitamin A reserves in the livers of three such animals indicated low total reserves of 90, 110 and 140i.u. respectively.

All the rats were given our standard vitamin A-deficient

Table 1. *Average total, free and bound protease activities in livers of female rats given various intakes of vitamin A (series 1 experiments)*

The free and bound acid-protease activities of rat liver were estimated in animals maintained on diets containing known amounts of vitamin A. Vitamin A content of the liver was estimated and expressed as i.u./g. wet wt. Lysosome stability was measured as the free activity expressed as a percentage of the free plus bound activities, as described in the text, after the tissue had been disintegrated in an all-glass homogenizer.

Group	No. of rats	Mean time on diet (days)	Mean time on excessive vitamin A* (days)	Body wt. (g.) Initial	Maximum	Final	Vitamin A (i.u./g.)	Activity (μg. of tyrosine/hr./mg. wet wt.) Total	Free	Bound	Free activity (% of free + bound activities)
1. Deficient (no vitamin A)	5	51	—	69	169	151	2	2·94	0·12	1·56	6·7
2. Marginal (25i.u. of vitamin A weekly)	5	41	—	71	182	182	13	3·15	0·21	1·47	12·9
3. Adequate (150i.u. of vitamin A weekly)	5	42	—	72	193	193	170	2·37	0·18	1·98	8·2
4. Excessive (50000 i.u. of vitamin A daily)	6	48	26	156	162	150	18000	4·20	0·60	1·71	25·7

* Included in previous column.

Table 2. *Average total, free and bound protease activities in kidneys of female rats given various intakes of vitamin A (series 1 experiments)*

Experimental conditions were as described in Table 1.

Group	No. of rats	Mean time on diet (days)	Mean time on excessive vitamin A* (days)	Activity (μg. of tyrosine/hr./mg. wet wt.) Total	Free	Bound	Free activity (% of free + bound activities)
1. Deficient (no vitamin A)	5	51	—	5·19	0·78	1·89	28·5
2. Marginal (25i.u. of vitamin A weekly)	5	41	—	5·52	1·44	1·89	43·3
3. Adequate (150i.u. of vitamin A weekly)	5	42	—	4·47	0·99	1·32	42·1
4. Excessive (50000i.u. of vitamin A daily)	6	48	26	6·30	1·44	1·92	40·9

* Included in previous column.

Table 3. *Total, free and bound protease activities in the livers of male rats given various intakes of vitamin A (series 2 experiments)*

The experimental conditions were as described in Table 1, except that tissue was disintegrated in a Perspex-and-glass homogenizer (Aldridge *et al.* 1960).

Group	Rat no.	Time on diet (days)	Body wt. (g.)				Liver wt. (g.)	Vitamin A (i.u./g.)	Activity (μg. of tyrosine/hr./mg. wet wt.)			Free activity (% of free + bound activities)
			Initial	Maximum	Final	Loss			Total	Free	Bound	
5. Deficient (no vitamin A)	22	46	55	218	195	23	6·7	1	2·64	0·39	1·50	21
	23	48	67	243	186	57	4·8	2	4·17	1·44	1·83	44
	24	49	65	242	210	32	7·2	1	2·27	0·18	1·68	10
	25	50	64	214	188	26	6·8	1	1·98	0·03	1·32	2
	26	50	64	223	212	11	9·3	1	2·82	0·27	1·89	13
	27	52	63	262	213	49	6·7	1	2·46	0·15	1·53	9
	28	53	56	214	160	54	5·8	1	2·82	1·20	1·35	47
	29	55	51	226	194	32	6·9	1	3·66	0·69	2·10	25
	30	60	61	249	165	84	5·3	1	3·30	0·78	1·59	33
	31	60	57	261	192	69	7·0	1	2·91	0·78	1·32	37
	32	60	57	238	176	62	5·6	1	4·08	1·20	1·41	46
	33	62	72	256	237	19	9·2	1	4·59	0·57	2·19	21
Average		54	61	237	194	43	6·8	1	3·14	0·64	1·64	26
6. Marginal (25 i.u. of vitamin A weekly)	34	48	67	255	255	0	12·9	1	2·43	0·18	1·26	13
	35	49	62	265	265	0	14·4	1	1·02	0·00	0·87	0
	36	51	65	297	297	0	13·6	1	1·77	0·03	1·32	2
	37	51	61	299	299	0	13·6	1	1·98	0·15	1·32	10
	38	52	63	248	243	5	6·6	1	2·79	0·24	1·80	12
	39	77	67	327	327	0	12·7	145	2·46	0·33	1·59	17
	40	83	56	274	262	12	8·0	1	2·58	0·57	1·44	28
	41	86	82	334	334	0	14·3	282	1·86	0·00	1·17	0
	42	88	65	312	312	0	11·1	1	1·53	0·00	1·29	0
	43	88	63	338	338	0	13·8	1	1·59	0·00	1·47	0
	44	88	60	318	318	0	12·5	1	1·80	0·00	1·68	0
	45	90	58	303	303	0	11·9	1	3·54	1·02	1·65	38
	46	90	55	294	294	0	11·7	1	3·15	0·06	1·98	3
	47	90	51	261	261	0	9·4	1	2·94	0·00	1·65	0
Average		74	63	295	293	1·2	11·9	31	2·25	0·18	1·46	9

		Days on excessive vitamin A										
7. Excessive (50000i.u. of vitamin A daily)	48	43	172	186	150	36	7·7	6980	6·54	1·41	0·96	59
	49	45	148	167	159	8	7·6	6200	4·05	1·59	1·38	54
	50	48	154	241	241	0	12·0	13300	4·62	2·28	1·50	60
	51	50	173	242	238	4	10·8	5500	3·30	1·11	1·29	46
	52	50	143	184	169	12	8·0	20000	3·06	1·11	1·32	46
	53	55	194	223	199	24	8·6	8240	4·65	0·75	0·63	54
	54	55	132	168	156	12	8·1	7630	4·26	1·23	1·20	51
	55	57	166	235	235	0	11·5	12300	4·56	1·50	1·32	53
	56	77	153	198	173	25	8·0	4740	4·02	1·11	1·20	48
	57	86	165	199	194	5	7·0	1980	4·92	0·87	0·81	52
	Average	57	160	204	191	13	8·9	8690	4·40	1·30	1·16	52

diet, containing: casein (vitamin-free), 20 parts; sucrose, 60; arachis oil, 15; dried yeast, 10; minerals, 5. The mineral mixture was made up of the following: $Ca_3(PO_4)_2$, 400 parts; KCl, 250; NaH_2PO_4, 105; $MgSO_4$, 80; NaCl, 50; iron citrate, 35; KI, 1·0; $MnSO_4$, 0·2; NaF, 0·04. Adequate weekly doses of vitamins D, E and K were also allowed: these vitamins were dissolved in a little arachis oil and administered orally, separately from the rest of the diet.

Arrangements for the dosing of groups with vitamin A varied in detail between successive experiments, but the intended vitamin A status and the level of dosing were always chosen from the following four gradings of vitamin A status: 'deficient', no vitamin A given; 'marginal', 25i.u. of vitamin A weekly; 'adequate', 1000i.u. of vitamin A weekly; 'excessive', 50000i.u. daily.

Vitamin A was always given in the form of its synthetic acetate, dissolved in arachis oil. At the lower levels the dose was administered into the rat's mouth by a dropping-pipette. At the excessive level the vitamin A was mixed into the diet. To avoid deterioration of the vitamin fresh supplies of diet were made up daily; a solution of vitamin A in the bulk of the arachis oil was mixed with the premixed solid components of the diet. In most of the rats given toxic excess of vitamin A the incidence of the skeletal fractures, typical of hypervitaminosis A, was studied by radiography (Moore & Wang, 1945).

Series 1 *experiments.* Four groups, each of at least ten female rats, were restricted to the diet deficient in vitamin A at body weights of about 70g. Groups 1, 2 and 3 received doses of vitamin A equivalent to 0, 4 or 150i.u. daily. The rats were kept on their diets for different periods, as shown in Table 1. Most of the animals in group 1 stopped growing and declined in body weight, usually with the development of xerophthalmia. In this group only those rats that showed clear signs of deficiency were used for the preparation of subcellular fractions.

The action of toxic excess of vitamin A in arresting growth in rats is much more rapid than that of dietary deficiency. Therefore, to prevent a great disparity in size and age between the avitaminotic and hypervitaminotic animals, the commencement of toxic overdosing in group 4 was delayed until day 20 of the experiment. Until this point the rats received 4i.u. of vitamin A daily.

The timing of the experiment was made difficult by the laborious procedure necessary for the preparation of the tissue fractions, which did not permit more than two rats to be used on the same day. Some rats in groups 1 and 4 were therefore wasted because they died before they could be used in the way intended. In this series both the liver and kidneys were fractionated (Tables 1 and 2).

Series 2 *experiments.* In further experiments male rats were used. The group given 'adequate' doses of vitamin A (150i.u. daily) was omitted. To avoid too many rats becoming available for use at once, the animals were started on their diets in three batches. As far as possible the killing of rats of the same group in succession was avoided. The periods during which the animals received their diets, and their changes in body weight during the experiment, are summarized in Table 3. In this series only the liver was fractionated.

Control experiments on starved or pair-fed rats. In Expt. 3 (Table 4) six young male rats, weights approx. 75g., were divided into equal groups. One group was starved for 24hr. before being killed, the other group was not starved.

In Expt. 4 (Table 5) 12 adult male rats, weights 250–300g., were divided into four groups of three rats each. Two of the groups were starved for 24 or 48hr. respectively before being killed, the remaining groups serving as unstarved controls. In Expt. 5 (Table 6) four rats given toxic excess of vitamin A were matched with four others, which served as pair-fed controls.

Homogenization of tissues. Immediately after each rat had been killed by stunning and decapitation, the liver and sometimes also the kidney were removed and cooled for 5min. in 0·25M-sucrose at 4°. A 2·0g. sample of tissues was then taken for homogenization and was placed in 20ml. of ice-cold 0·25M-sucrose. In the experiments of series 1 an all-glass homogenizer was used. Trials on a number of samples of the same liver gave free proteolytic enzyme activities, as percentages of the total activity (see below), with a standard deviation of 15·3 on a sample mean of 14·4. Estimations of total enzyme activity gave a much lower standard deviation. The wide variations in free activity therefore were presumably not due to unevenness in the composition of the tissue samples, or to gross inaccuracies in the assay method, but to low efficiency in homogenization. For all our subsequent experiments a homogenizer of the type described by Aldridge, Emery & Street (1960) was used. On 11 samples of the same tissue estimations the percentage of free enzyme activity had a standard deviation of only 4·9 on a sample mean of 37·9. The clearance of the Perspex piston in the glass homogenizer did not vary, and little or no wear was seen after months of use.

Preparation of tissue fractions. For the estimation of 'total' protease activity 1ml. of the homogenate was taken, and its bound enzyme released by the addition of 0·5ml. of an aqueous 1% solution of the detergent BRIJ 35, together with 1ml. of water. The remainder of the homogenate was then spun in 50ml. tubes in an MSE refrigerated centrifuge (rotor no. 69181) at 600*g* for 5min. to remove nuclei and debris. The pellet was discarded and the supernatant was respun at 15000*g* for 20min. 'Free' acid-protease activity

Table 4. *Average total, free and bound protease activities in livers of young male rats starved for 24hr. compared with unstarved controls (series 3 experiments)*

Estimations of total, free, and bound protease activities were carried out as described in the text after starvation for 24hr. Animals of the same age and sex were used as controls.

Group	No. of rats	Body wt. (g.): On day before being killed	Body wt. (g.): On day killed	Liver wt. (g.)	Activity (μg. of tyrosine/hr./mg. wet wt.): Total	Free	Bound	Free activity (% of free+bound activities)
8. Starved	3	81	73	3·15	5·90	0·75	3·53	18·1
9. Controls, not starved	3	80	86	4·27	3·43	0·22	2·30	8·6

Table 5. *Average total, free and bound protease activities in the livers of adult male rats starved for 24 and 48hr. compared with unstarved controls (series 4 experiments)*

Experimental conditions were as described in Table 4, except that some animals were starved for 48hr.

Group	No. of rats	Body wt. (g.): On day before being killed	Body wt. (g.): On day killed	Liver wt. (g.)	Activity (μg. of tyrosine/hr./mg. wet wt.): Total	Free	Bound	Free activity (% of free+bound activities)
10. Starved for 24hr.	3	272	256	8·1	6·03	0·66	3·21	16·6
11. Starved for 48 hr.	3	287	275	7·3	6·87	0·69	3·63	16·3
12. Controls, not starved	6	280	286	10·2	3·69	0·51	2·25	16·8

Table 6. *Average total, free and bound protease activities in the livers of hypervitaminotic rats compared with pair-fed controls (series 5 experiments)*

Protease activity was measured in the livers of hypervitaminotic animals and pair-fed controls; the results are the averages for four animals in each group. Rats received the experimental diet for periods ranging from 9 to 31 days.

Group	Body wt. (g.): Maximum	Body wt. (g.): Final	Activity (μg. of tyrosine/hr./mg. wet wt.): Total	Free	Bound	Activity (% of free+bound activities)
13. Hypervitaminotic A	100	90	4·55	0·59	2·44	19·8
14. Controls	110	106	4·93	0·32	2·99	9·9

Table 7. *Stability of lysosomal enzymes in liver slices from rats of series 2 experiments*

Rat liver from control and hypervitaminotic animals was sliced on a tissue chopper and incubated in Krebs–Ringer buffer, pH 7·4, for 30 min. at 37°. The tissue was then homogenized and free and total activities of acid protease were estimated in the usual manner. Significance of differences: unincubated, $0{\cdot}001 < P < 0{\cdot}01$, i.e. highly significant; incubated, $P < 0{\cdot}001$, i.e. very highly significant.

	Controls		Hypervitaminotic A	
	Unincubated	Incubated	Unincubated	Incubated
Free activity (% of total activity)	18·4	41·2	39·3	70·8
Based on no. of animals	11		7	

was estimated in the supernatant, without further treatment. In this paper 'free' activity is defined as that enzyme which does not sediment with particulate material at 15000*g*. For the estimation of 'bound' activity the pellet was resuspended by homogenization with 18·4 ml. of 0·1% BRIJ 35. The 0·1% BRIJ was found to give maximum release of bound activity. No effect of the detergent on the activity of solubilized enzyme was observed up to 0·5%.

Estimation of enzyme activity. The activity of the fractions (1 ml.), prepared as described above, was measured after the addition of 1 ml. of a 4% solution of haemoglobin in 0·1 M-acetate buffer, pH 4, as substrate. The solutions were incubated for 20 min. at 37°. Then 5 ml. of 4·7% (w/v) trichloroacetic acid was added and the precipitate was removed by filtration. Free 'tyrosine' was estimated in the filtrate by means of the phenol reagent of Folin & Ciocalteu (1927). Unincubated controls were prepared for each estimation. The enzyme activity was expressed as μg. of tyrosine liberated/hr./mg. wet wt. of the original tissue. A tyrosine standard was used for each batch of estimations.

Effect of slicing liver, and of the incubation of lysosome preparations, on enzyme activity. To explore further the effect of hypervitaminosis A on the stability of the lysosomes post-mortem experiments of two types were made on the livers of some of the rats.

(1) The liver was sliced on a tissue chopper (McIlwain & Buddle, 1953) and incubated in Krebs–Ringer buffer, pH 7·4, for 30 min. at 37°. This treatment has been shown by Dingle (1963) to increase the ratio of free to bound enzyme in the tissue. For comparing the extent of this effect in hypervitaminotic and control rats measurements were made of the free enzyme activity, expressed as a percentage of the total activity, both before and after incubation. The animals used were from series 2 (Table 7).

(2) A granule fraction, rich in lysosomes, was prepared as described in Table 8 (group 4) from hypervitaminotic and control rats (series 2). The particles were suspended in 0·25 M-sucrose and incubated at 37°. At intervals portions were removed and sedimented, and free protease was measured in the supernatant. The free enzyme was again expressed as a percentage of the total activity (Fig. 1).

Estimation of vitamin A. To check that the graded dietary intakes of vitamin A had the desired effects on the vitamin A contents of the tissue, 1–2 g. samples of liver and sometimes also of kidney were taken from each rat. The tissues were finely chopped with scissors; 2 ml. of ethanol was then added, followed by 0·5 ml. of aqueous saturated KOH. The

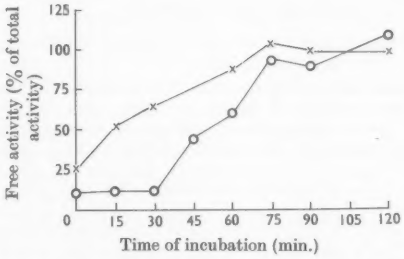

Fig. 1. Release of protease from isolated rat-liver lysosomes. ○, Control; ×, hypervitaminotic A. A granule fraction rich in lysosomes was prepared from livers of hypervitaminotic and control rats. Particles were suspended in sucrose, incubated at 37°, and at intervals the non-sedimentable acid protease was measured. The results at each time are expressed as a percentage of the total activity of the suspension.

mixture was heated in a boiling tube until the tissues were dissolved (5–10 min.), and was cooled and extracted twice with 20 ml. portions of ether. The ether extracts were combined, washed with water, dried with anhydrous Na_2SO_4 and evaporated on a hot-water bath under reduced pressure. The residue was dissolved in chloroform, and portions of the chloroform solution were taken for treatment with the $SbCl_3$ reagent, the blue colour being measured in a photoelectric absorptiometer, fitted with a Wratten filter no. 26. The absorptiometer had previously been calibrated with vitamin A acetate. The extraction procedure followed that of Davies (1933).

Distribution of vitamin A in tissue fractions. Several experiments were made on the distribution of vitamin A in the subcellular fractions. Since information was desired on the relative vitamin contents of the various fractions, the method of fractionation was different from that used for the sole purpose of estimating the lysosomal enzymes. Combined livers from four rats from Expt. 1 (group 4) were minced and a sample was taken. The remainder was homogenized in 0·4 M-sucrose and centrifuged in successive portions of suitable size. Nuclei and cell debris were removed by spinning for 5 min. at 600*g* (fraction 1).

Table 8. *Distribution of vitamin A in various subcellular fractions of the pooled livers of hypervitaminotic rats (group 4, series 1 experiments)*

Experimental details are given in the text.

Fraction		Vitamin A		
No.	Nature	(i.u./g. of original liver)	(% of total vitamin A in fraction)	(i.u./mg. of protein N in fraction)
	Whole liver	12800		471
	Homogenized and spun			
1.	Spun at 600*g*: pellet of nuclei and debris	4900	38·3	1100
2.	Respun at 15000*g*: supernatant (soluble material and ribosomes)	5700	44·5	820
	Pellet from 15000*g* homogenized			
3.	Spun at 4500*g*: pellet of mitochondria	840	6·6	620
4.	Respun at 8000*g*: pellet of lysosomes etc.	30	0·2	130
5.	Soluble material and ribosomes	6	0·05	5

Spinning at 15000*g* for 20 min. followed and the supernatant was removed (fraction 2). The combined 15000*g* pellets were rehomogenized with sucrose solution, layered over 0·6M-sucrose and centrifuged first at 4500*g*, giving a pellet mainly of mitochondria (fraction 3). Further layering and centrifugation at 8000*g* produced a pellet that on electron-microscope observations was found to contain lysosomes, small mitochondria and a few ribosomes (fraction 4), and a supernatant containing soluble and emulsified material and some ribosomes (fraction 5).

The estimations of vitamin A were difficult owing to the large volumes of the supernatant fractions that had to be extracted and to the presence of sucrose. Various minor modifications of the method of extraction of the vitamin had therefore to be made. Even with these modifications, however, attempts to study the distribution of the vitamin in the livers of the rats given marginal or adequate doses of vitamin A failed. Serious discrepancies were found between the total amount of the vitamin in the various fractions and in the original liver. A reasonably good recovery of vitamin A was obtained in the various fractions from hypervitaminotic liver (Table 8).

RESULTS

The numbering of the Tables in which our results are summarized has already been given. To save space results for individual rats are only given for our series 2 experiments (Table 3), which were the most successful as a demonstration of the effect of the vitamin A status on the lysosomal enzymes. In the other series of experiments average results are given.

DISCUSSION

Technical limitations. In Tables 1–6, our findings for proteolytic activities are given under the headings (1) total, (2) free and (3) bound. If we accept the view that almost all the acid-proteolytic activity of the organs under investigation originates in their lysosomes, which may not be far from the truth, then the total activity ought to be equal to the sum of the free and bound activities. The consistent tendency, in our results, for the total activity to exceed considerably the sum of the free and bound activities suggests that usually our homogenizer was only 50–70% efficient in breaking the liver cells. In view of the technical difficulties involved this low efficiency is not surprising. Presumably unbroken cells must have been included in our first pellet, obtained by low-speed centrifugation, which was discarded.

Changes in our homogenizer made between series 1 and series 2 appear to have improved its efficiency in disrupting the liver cells. The narrowing of the gap between the piston and cylinder of the homogenizer, however, had to be undertaken cautiously, so as to avoid unwanted breakages in the organelles, including the lysosomes, as this would cause an increase in the free proteolytic activity at the expense of the bound activity. In view of the incomplete breakage of the liver cells it seemed preferable to express the free proteolytic activity as a percentage of the sum of free and bound activities, as found experimentally, rather than as a percentage of the total activity. This percentage, i.e. (free × 100)/(free + bound), is considered to be a measure of the stability of the lysosomes to homogenization. The term 'free', as used in this connexion, does not imply that the enzyme is free in the cell cytoplasm but is an indication of the lability of the lysosomes to mechanical stress.

The high concentration of proteolytic activity in the liver lysosome-rich fraction is shown by the fact that, whereas this fraction contributed only about 1·4% of the nitrogen content of the whole liver, it contained at least 80% of the total proteolytic activity. The concentration of activity

in the lysosomes therefore must be about 60 times as great as that in the liver as a whole.

Our method for separating subcellular fractions was open to the criticism that the products of the homogenization of more than one type of cell were included. Thus the fractions from liver were doubtless derived mainly from parenchymal cells, but contributions were presumably also made by the Kupffer cells and those of the blood vessels and bile ducts. The possibility remains that variations in the vitamin A status may affect some types of liver cells more than others.

Increase of free proteolytic activity in the liver in hypervitaminosis A. In both series 1 and 2 the free proteolytic activity in the liver of those rats that were given toxic doses of vitamin A was much higher than in any of the other groups. In series 1, for example, this difference was statistically significant ($0{\cdot}001 < P < 0{\cdot}01$) between the hypervitaminotic group 4 and the deficient group 1, with means of 0·60 and 0·12 μg. of tyrosine/hr./mg. wet wt. respectively. In series 2 the free activity in the hypervitaminotic group 7 was also much greater than in the marginal group 6, with means of 1·30 and 0·18 μg. of tyrosine/hr./mg. wet wt. respectively. In both series the ratio of free activity to the sum of free and bound activities was increased in hypervitaminosis. Total activity was also raised in hypervitaminosis, but not to the same extent as the free activity.

In some of our hypervitaminotic rats the toxic effects of the vitamin on the lysosomes may have been augmented by those of emaciation. In at least some of the rats (e.g. nos. 50, 51, 55 and 57), however, large increases in free proteolytic activity occurred without appreciable loss of body weight, and in the presence of substantial amounts of intraperitoneal fat.

The experiments *in vitro* with sliced liver and with incubated lysosomes indicated that the fragility of the lysosomes towards both these treatments, as indicated by the liberation of their enzymes, was increased in hypervitaminosis A.

Proteolytic activity in the liver in avitaminosis A. Our results for avitaminosis A differed in the experiments of series 1 and 2. In series 1 no evidence of a rise in the free proteolytic activity was found in the avitaminotic rats, as compared with the activity in rats given marginal or adequate doses of vitamin A. In series 2, with much larger groups of animals, free activity was higher in the deficient than in the marginally dosed rats, with means of 0·64 and 0·18 μg. of tyrosine/hr./mg. wet wt. respectively. These findings seem in good agreement with those of Roels, Trout & Guha (1964), who reported that the free β-glycerophosphatase activity of rat-liver preparations is increased in 'frank' vitamin A deficiency but not in 'mild' deficiency. As judged by the percentage loss of body weight our rats were more severely deficient in series 2 than in series 1. Even in our series 2, however, the free activity was much lower in the deficient than in the hypervitaminotic animals.

Proteolytic activity in the liver in starvation. The results of our experiments on young rats starved for 24 hr. (Table 4) confirmed the finding of Beaufay *et al.* (1959) that starvation may cause the release of lysosomal enzymes. In contrast, the starving of adult rats for 24 or 48 hr. (Table 5) did not increase the release of their enzymes. Possibly this difference was related to the presence of much larger fat deposits in the older animals.

The higher values for free proteolytic activity in liver preparations from the control rats in Expt. 4 as compared with those from the controls, in Expts. 1, 2 and 3, may possibly have been due to their greater age. In rat liver and kidney Franklin (1962) has demonstrated an increase in the percentage of free lysosomal enzyme activity in the first 2 months of life, and in kidney has reported a similar increase as the animals become senile. These results suggest that further studies on the effect of age on lysosome lability are desirable.

The results of Expt. 5 (Table 6) indicate that hypervitaminosis A has a greater effect on the release of lysosomal enzymes than can be explained by the associated voluntary reduction in food intake. It was noticed, however, that the stomachs of the hypervitaminotic rats invariably contained substantial amounts of undigested food, and that the intestinal contents and faecal pellets were putty-coloured. In contrast, the stomachs of the control pair-fed rats were empty, and their intestinal contents and faeces were normal in colour. In this experiment therefore pair-feeding produced equality in the amount of food ingested rather than in the amount of food digested. No simple method of avoiding this complication seems available.

Proteolytic activity of kidneys. In series 1 there was no clear evidence that the vitamin A status affected the proteolytic activity of the kidneys. Values for total and free activities, but not for bound activity, were consistently higher in the kidneys than in the liver of rats of the same series.

Distribution of vitamin A in relation to proteolytic activity. The results in Table 3 indicate that, on the basis of either the total vitamin A content or its concentration per mg. of nitrogen, there was no evidence of a concentration of vitamin A in the lysosome fraction. Indeed, in contrast with the great concentration of proteolytic activity in the lysosomes, it appears that the vitamin is less concentrated in these organelles than in the liver as a whole. This conclusion, of course, refers to the whole lysosomes. It remains possible that the

vitamin may be highly concentrated in the lysosomal membrane.

General. Our results show clearly that the lysosomes of rat liver are more unstable in animals that have been made hypervitaminotic than in control animals. This finding agrees with the action *in vitro* of vitamin A on isolated lysosomes (Dingle, 1961; Fell, Dingle & Webb, 1962); it is also consistent with the effect of the vitamin on skeletal tissues in organ cultures (Fell & Dingle, 1963), and with the hypothesis that the changes seen in hypervitaminosis A in the living animal are associated with the release of bound hydrolytic enzymes (Lucy, Dingle & Fell, 1961). In studies on hypervitaminosis A induced by dosing with vitamin A acid, which differs widely from vitamin A alcohol and esters with regard to its transport and storage in the body, Weissmann & Thomas (1963) have reported increased lysosomal activity in the livers of rabbits, and Weissmann, Uhr & Thomas (1963) in the livers of guinea pigs.

Our findings on rats deficient in vitamin A also support the view that this condition may affect the stability of the lysosomes, although the injury to the lysosomes seems less severe than in hypervitaminosis.

It must be emphasized, however, that the activity of lysosomes can be affected by many other factors besides the vitamin A status. Thus we have confirmed that starvation may raise enzyme activity, as reported by Beaufay *et al.* (1959), although this effect seems smaller and less consistent than in hypervitaminosis A. The increases in free enzyme activity sometimes found in avitaminosis A may probably be partially explainable on this basis. Cortisone and hydrocortisone have been repeatedly implicated as antagonists to vitamin A in its effect on lysosomal activity (Weissmann & Thomas, 1963) or on organ cultures (Weissmann & Fell, 1962). Increased lysosomal enzymes have been found by Tappel, Sawant & Shibko (1963) in various forms of muscular dystrophy, including those induced by avitaminosis E. Dingle *et al.* (1963) have mentioned that the lysosomal cathepsin of rat kidneys is increased in deficiency of vitamin E.

No explanation can yet be given why, if both starvation and hypervitaminosis A can cause the release of enzymes from lysosomes, skeletal fractures are caused only by hypervitaminosis A. Presumably this question will only be answered when more is known about the relative vulnerability to vitamin A of lysosomes in different tissues, and about other relevant factors.

Our thanks are tendered to Dame Honor B. Fell and Dr E. Kodicek for interest and advice, and to Mr J. C. Hanlon and Miss Patricia Carter for technical assistance. J. T. D. is a member of the External Staff of the Medical Research Council.

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Biochem. J. (1966) **98**, 485

Minor Proteases in the Stomach of the Pig

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(*Received* 3 *August* 1965)

1. Pepsins C and D and probably pepsin B as well as the major enzyme pepsin have been detected on starch-gel electrophoretograms of concentrates of the gastric contents of four pigs. 2. The presence of pepsin B is further indicated by a high ratio of peptidase activity to haemoglobin-digesting activity after treatment at pH 6·9.

Previous reports have described the isolation of three minor gastric proteolytic zymogens, pepsinogen B (Ryle, 1965*a*), pepsinogen C (formerly called parapepsinogen II) (Ryle, 1960) and pepsinogen D (Lee & Ryle, 1963; Ryle, 1965*a*), from the gastric mucosa of the pig and the isolation of pepsins B and C (formerly parapepsins I and II) from crude commercial pepsin (Ryle & Porter, 1959). The third minor enzyme, pepsin D, has also been found in crude and crystalline commercial pepsin preparations (D. Lee & A. P. Ryle, unpublished work).

Since commercial pepsin preparations are apparently made by acid extraction of the gastric mucosa, the reports quoted above do not provide any evidence that the minor zymogens, or their corresponding enzymes, are secreted into the stomach. This paper presents evidence that the minor enzymes are, as expected, found in the stomach contents.

EXPERIMENTAL

Materials. Haemoglobin was bovine haemoglobin enzyme substrate powder (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex). Acetyl-L-phenylalanyl-L-di-iodotyrosine was prepared as described by Baker (1951) and had m.p. 231° (decomp.) and $[\alpha]_D^{20} + 30{\cdot}5 \pm 0{\cdot}8°$ (*c* 2 in pyridine).

Hydrolysed starch was obtained from Connaught Medical Research Laboratories, Toronto, Canada.

Dialysis tubing (Visking) was obtained from Hudes Merchandising Corp. Ltd., London, W. 1.

Polyethylene glycol (Carbowax 20M), mol.wt. 15000–20000, was obtained from Union Carbide Ltd., London, W. 1.

Preparation of samples. The stomachs were removed from castrated male bacon pigs about 5 months old as soon as possible after slaughter, i.e. 15–20min. after electrical stunning, and the contents were emptied into jars kept on ice for transport to the laboratory. The pH of the contents was measured, the total contents were then filtered with the aid of Hyflo Super-Cel and the filter was washed with 0·1M-acetate buffer, pH 5·6. The filtrate and washings were combined and concentrated to about 20ml. by dialysis in 24/32in. Visking tubing against polyethylene glycol and the proteolytic activity against haemoglobin was then measured.

The activation of any remaining zymogens was now completed by acidification to pH 2 with dilute HCl for 30min. at room temperature. The pH was then readjusted to 5 by addition of M-acetate buffer, pH 5·6, and the preparations were dialysed against 0·1M-acetate buffer, pH 5·6, and finally concentrated by dialysis against polyethylene glycol in 9/32in. Visking tubing to give about 1ml. of dark-green solution.

Although polyethylene glycol is known to enter the dialysis sac (Ryle, 1965*b*) its presence was found not to interfere with the activity determinations and electrophoresis.

Electrophoresis in starch gel. Samples (0·01ml.) of the concentrated preparations were applied to segments (9mm. × 5mm.) of Whatman 3MM filter paper, which were then inserted into a horizontal gel prepared in the usual way beside samples of pepsin and of pepsins B, C and D, all of which were 2mg./ml. After the electrophoretic run the gel was sliced horizontally and the two cut surfaces were used for detection of the enzymic activities.

Detection of activity with haemoglobin. The method used is based on that of Uriel (1960). The gel was covered with a piece of Whatman no. 1 filter paper moistened with a mixture of 2·5% haemoglobin and 0·315N-HCl (4:1, v/v). The paper was removed after 20min. and after a further 20min. at room temperature the gel was stained for protein with Naphthalene Black 10B (G. T. Gurr Ltd., London, S.W. 6) in the usual fashion. The enzymes are detected as a white area on a blue ground.

Detection of activity with gelatin. A warm solution of 5% gelatin in 0·05N-HCl containing sufficient Congo Red to give a deep-purple–blue colour was poured on to the gel and allowed to set. After 15–20hr. at room temperature the gel was gently washed under the cold tap, leaving white areas where the enzymes had liquefied the gelatin. On the original gel these areas were clearly distinguishable from the light areas caused by the uneven thickness of the gelatin layer.

Determination of activity with acetyl-L-phenylalanyl-L-di-iodotyrosine and haemoglobin. These assays were carried out as described by Ryle (1965*a*) and Ryle & Porter (1959)

respectively. The units used are μmoles of di-iodotyrosine liberated/min. and proteolytic units ([P.U.]^{Hb}).

RESULTS

Preparation of samples. Four stomachs were examined; the total activity found in each was about 0·8 [P.U.]^{Hb}, corresponding to 30–40 mg. of pepsin, so that the solutions applied to the starch gel were equivalent to 30–40 mg. of pepsin/ml. This is much more than is necessary for the detection of a single enzyme but a considerable overload was used to allow the detection of minor components. The pH of the stomach contents varied from 4·5 to 5·1, and since it was thus possible that they contained incompletely activated zymogens they were treated at pH 2 at room temperature for 30 min. to complete the activation before electrophoretic examination.

Electrophoresis in starch gel. In preliminary experiments I failed to find any conditions in which all four enzymes could be separated in a single run. Pepsin and pepsin D can be separated by electrophoresis at pH 3·2 but at this pH the separation of pepsins B and C from one another is marginal. These two enzymes can be separated from one another at pH 5·6, when they migrate towards the anode (Ryle & Porter, 1959), but it was found that the faster-migrating pepsin in the concentrates left a trail that interfered with the detection of pepsins B and C. It was decided to use pH 3·2 for separation of pepsin, pepsin D and pepsins B plus C and to rely on the differential specificity and stability of the enzymes for the identification of the last pair.

Figs. 1 and 2 show the results from a single run. In Fig. 1 it is apparent that the pepsin and pepsin D standards are each contaminated with the other but the enzymes are detected at 1·8 and 0·7 cm. in the direction of the anode respectively. Pepsin B has little or no activity against haemoglobin, the weak activity being possibly due to contaminating pepsin C. All four samples of stomach contents clearly show pepsin C at 7 cm. in the direction of the cathode, and despite the heavy load of pepsin it was just possible in the original to detect pepsin D in all four samples as well.

In Fig. 2 the presence of pepsin and pepsin D is clear in all four samples. On the cathodic side of the origin there is a suggestion of two zones of activity of which the slower, more intense one would be due to pepsin B and the faster, weak one due to pepsin C, which has about one-fifth the gelatin-liquefying activity of pepsin B as measured by fall in viscosity of a solution (Ryle & Porter, 1959). However, the separation of pepsin B from pepsin C under these conditions is too slight to allow the certain identification of pepsin B in the presence of pepsin C.

Judged by the intensity of the spots of pepsin C and pepsin D these enzymes may each be about 1% of the total.

Activity after treatment at pH 6·9. Pepsin C has little or no activity with acetyl-L-phenylalanyl-L-di-iodotyrosine as substrate at pH 2 and is slowly inactivated at pH 6·9 (Ryle & Porter, 1959), whereas pepsin B attacks this substrate at pH 2 and is stable at pH 6·9. Both pepsin D (D. Lee & A. P. Ryle, unpublished work) and pepsin are rapidly inactivated at pH 6·9. Thus pepsin B can be identified by its activity (stable at pH 6·9) with the acetyl-peptide as substrate at pH 2.

Samples of the concentrated stomach contents were dialysed against 0·02 M-phosphate buffer, pH 6·9, in the presence of toluene to inhibit bac-

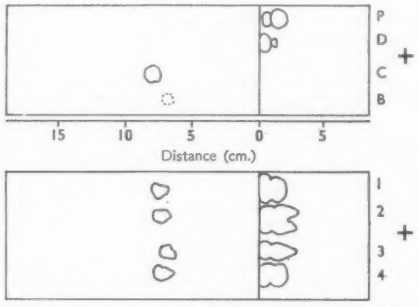

Fig. 1. Starch-gel electrophoresis of concentrates of gastric contents. The conditions used were: 0·05 M-citrate buffer, pH 3·2; 2·5 v/cm.; 22 hr. Activity was detected with haemoglobin. B, C, D and P: Markers of pepsins B, C and D and of pepsin; 1–4: four concentrates. A tracing of a photograph is shown.

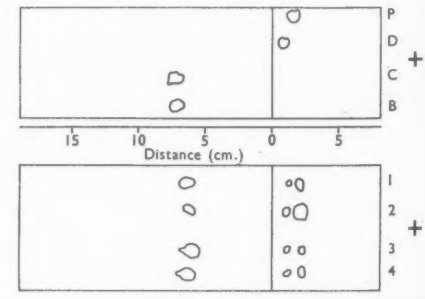

Fig. 2. Starch-gel electrophoresis of concentrates of gastric contents. The conditions and symbols were as defined in Fig. 1, but the activity was detected with gelatin. A tracing of a photograph is shown.

terial growth for 24hr. at 2° and then for 39hr. at room temperature. A precipitate was removed from each by centrifugation and the activities of the supernatants against the acetyl-peptide and against haemoglobin were determined.

The ratios (μmoles of di-iodotyrosine liberated/min./$[P.U.]^{Hb}$) found were 41, 46, 53 and 90, whereas the value for pepsin or pepsin D is 6·8–7·0. The activity of the samples against haemoglobin was decreased to less than 0·1% of the initial value but it is not clear why even this small activity remained. It is possible that one or more of the enzymes is stabilized by the presence of some other material in the concentrated gastric contents.

If the activity against the acetyl-peptide after treatment at pH 6·9 is correctly ascribed to pepsin B the amount found indicated that this enzyme accounted for at least 0·15–0·5% of the total enzyme in the stomachs. The value would be higher if any activity was lost with the precipitate removed by centrifugation.

DISCUSSION

All the proteolytic components detected in concentrates of pig stomach contents by electrophoresis in starch gel or by their specificity and stability properties can be accounted for as one or another of the known enzymes pepsins B, C or D or pepsin itself. All four have been detected shortly after death of the animals in the stomach of each of the four pigs examined although the amounts (relative to pepsin) of the minor components pepsins B, C and D appear to be rather less than those (4, 12 and 6% respectively; Ryle, 1965*a*) of the corresponding zymogens found in neutral extracts of the gastric mucosa. However, the estimate of the relative amounts of the enzymes made here is not at all accurate.

It appears likely that the three minor zymogens are secreted into the stomach during life although the possibility has not been excluded that they are only liberated during the trauma of death.

This work forms part of a programme supported by a grant from the Agricultural Research Council.

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Studies on Flavonoid Metabolism

METABOLISM OF FLAVONE IN THE GUINEA PIG

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(*Received 5 August* 1965)

1. The metabolism of flavone has been studied in the guinea pig: after administration of the compound either orally or intraperitoneally, 4′-hydroxyflavone is excreted in the urine. 2. Small amounts of 3′,4′-dihydroxyflavone were also detected in the urine after oral administration of flavone. 3. Hydroxyflavone formation is not suppressed by administering high doses of the antibacterial compounds aureomycin and phthaloylsulphathiazole. 4. Although salicylic acid appeared in trace quantities in the urine after administration of flavone, no other aromatic acid metabolites of flavone were detected.

Previous studies on the degradation of flavonoid compounds in the mammal (Booth, Murray, Jones & De Eds, 1956; Booth, Jones & De Eds, 1958*a*,*b*; Griffiths, 1962, 1964) have largely been limited to flavonoid compounds showing a high degree of hydroxylation. It was therefore decided to determine whether the non-hydroxylated parent compound, flavone, is absorbed and metabolized similarly in the mammal.

Administration of naturally occurring flavonoids showing hydroxylation of both ring A and ring B results in the formation of a number of phenolic acid metabolites, which are excreted in the urine. Although certain of these flavonoid metabolites may be products of mammalian metabolism (Booth *et al.* 1956), others have been shown to be formed largely by the microflora of the mammalian intestine (Griffiths, 1964). If flavone undergoes a similar type of ring-fission to that reported for naturally occurring flavonols (Booth *et al.* 1956) the formation of substantial amounts of non-phenolic aromatic acid metabolites from the cinnamoyl portion of the flavone molecule would be expected.

EXPERIMENTAL

Animals. Male guinea pigs each weighing approx. 300g. were used. The animals were housed in separate metabolism cages, designed to permit the separate collection of urine and faeces. The animals were fed at 24hr. intervals before the period of urine collection and unrestricted water intake was allowed throughout the experimental period.

Diet. The guinea pigs were fed on a standardized powder diet of the following composition: skimmed milk powder (fortified with vitamins A and D), 300g.; glucose, 170g.; potato dextrin, 150g.; potato starch, 234g.; arachis oil, 50ml.; vitamin fortification mixture (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), 23g.; cellulose powder, 100g.; salt mixture no. 4 (Hegsted, Mills, Elvehjem & Hart, 1941), 25g. Ascorbic acid (6mg.) was given to each guinea pig daily in aqueous solution.

Although adoption of this diet permits the elimination of plant materials containing flavonoid compounds from the diet it was not adequate for long-term feeding of the animals and was used only over experimental periods of short duration.

Test substance. Flavone, m.p. 99°, was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks. The compound was chromatographically pure. For oral administration flavone was given in admixture with the diet at the doses stated. For intraperitoneal administration, flavone was dissolved in 1ml. of acetone.

Preparation of urinary extracts for chromatography. Collection of the urine followed by ether extraction and paper chromatography of the ethereal extracts was carried out by our standard procedure (Griffiths, 1964).

Spray reagents. The reagent of Barton, Evans & Gardner (1952), diazotized *p*-nitroaniline and diazotized sulphanilic acid (Smith, 1960) were used for the detection of phenolic compounds.

A *p*-dimethylaminobenzaldehyde reagent (Smith, 1960) was used for the detection of aroyl-glycines.

Organic acids were detected by the use of an aqueous solution of 8-hydroxyquinoline sulphate (0·7%) and $ZnSO_4,7H_2O$ (0·5%). After spraying the spots were viewed in ultraviolet light (Pesez & Ferrero, 1957). Organic acids were also detected by the use of ethanolic 0·1% bromocresol green (Smith, 1960).

Flavone was detected by the use of 0·5% dinitrophenylhydrazine in 2N-HCl.

A saturated solution of ammonium molybdate was used for the detection of *o*-dihydroxyphenols.

Solvent systems. Two two-dimensional solvent systems

were used: *A*, propan-2-ol–aq. NH_3 (sp.gr. 0·88)–water (8:1:1, by vol.) followed by benzene–acetic acid–water (6:7:3, by vol.); *B*, chloroform–acetic acid–water (2:1:1, by vol.) followed by 20% (w/v) KCl.

Ultraviolet spectra. Ethanolic solutions of flavones and phenolic acids were examined in a Spectronic 505 recording spectrophotometer.

Infrared spectra. Hydroxyflavones (10mg.) in KBr (200mg.) disks were examined at 40% transmission in a Jasco infrared spectrophotometer [model (IR)–S; Japan Spectroscopic Co. Ltd.].

Alkaline hydrolysis of 4′-hydroxyflavone. Approx. 1mg. of the hydroxyflavone in 10ml. of aq. 50% (w/v) KOH was heated under reflux for 3hr. The degradation products were obtained by acidification of the hydrolysate with 0·1N-HCl to pH1 followed by ether extraction.

Determination of 4′-hydroxyflavone. A method based on the absorption of the compound at 327mμ was employed. The ethereal extract (3ml.) from 100ml. of urine of flavone-fed guinea pigs was applied to washed Whatman no. 3MM paper as a band. Markers of 4′-hydroxyflavone were applied to each side of the main band. The paper was developed (40cm.) with the benzene–acetic acid–water solvent and the chromatogram, after being dried, was examined in ultraviolet light. The fluorescent band corresponding to the fluorescent markers was cut into small fragments and eluted with acetone–ethanol (1:1, v/v) (3×10ml.). The extract was concentrated to 2ml. and applied to a second chromatogram of washed Whatman no. 3MM paper, which was developed with benzene–acetic acid–water solvent. The appropriate band was eluted with 3×7ml. of redistilled A.R. methanol. The extract after filtration was evaporated to dryness *in vacuo* and redissolved in 10ml. of redistilled A.R. ethanol, and this solution after suitable dilution was used for the spectrophotometric determination. The extinction at 327mμ was measured in a Hilger spectrophotometer. Standard curves were prepared by using a pure specimen of 4′-hydroxyflavone.

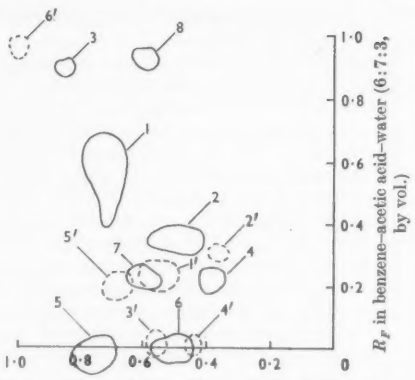

Fig. 1. Composite diagram showing the positions of metabolites of flavone and other aromatic constituents of guinea-pig urine in solvent system *A*. Experimental details are given in the text. Spots shown diagrammatically with a complete outline were detected by their fluorescence in ultraviolet radiation; spots shown with a broken outline were detected by the use of the appropriate spray reagent (see the text). Spot 1 (4′-hydroxyflavone): u.v. fluorescence, blue. Spot 2 (3′,4′-dihydroxyflavone): u.v. fluorescence, blue. Spot 3 (salicylic acid): u.v. fluorescence, blue. Spots 4–8 (unidentified): blue–dark-blue fluorescence. Spot 1′ (*p*-hydroxyphenylacetic acid): purple with diazotized *p*-nitroaniline. Spot 2′ (*p*-hydroxybenzoic acid): red with diazotized *p*-nitroaniline. Spot 3′ (*p*-hydroxyphenyl-lactic acid): purple with diazotized *p*-nitroaniline. Spot 4′ (*m*-hydroxyhippuric acid): red with diazotized *p*-nitroaniline. Spot 5′ (hippuric acid): orange–yellow with *p*-dimethylaminobenzaldehyde. Spot 6′ (flavone): orange with 2,4-dinitrophenylhydrazine, turning red after respraying with aq. 5% Na_2CO_3.

RESULTS

Aromatic acid constituents of urine of guinea pigs fed on the standard diet. Six guinea pigs were given the standard diet for 3 weeks and during the third week the urine was collected at 24hr. intervals. Examination of the ethereal extracts by two-dimensional paper chromatography indicated the presence of several phenolic compounds giving positive reactions with Barton's reagent and showing colour reactions with the diazotized *p*-nitroaniline reagent. These compounds were provisionally identified by co-chromatography as *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenyl-lactic acid and *m*-hydroxyhippuric acid (Fig. 1). The overall pattern closely resembles the phenolic acid excretion pattern of the rat fed on a diet based on white flour and dried milk (Griffiths, 1964). On spraying duplicate chromatograms with the *p*-dimethylaminobenzaldehyde reagent, an orange–yellow spot appeared that was shown by co-chromatography to be hippuric acid. Examination of the extracts by ascending chromatography with propan-2-ol–aq. ammonia–water revealed the presence of small amounts of benzoic acid (R_F 0·52). The latter compound was detected with the bromocresol green reagent and the 8-hydroxyquinoline sulphate reagent.

Metabolites of flavone. Twelve guinea pigs were given the standard diet for 2 weeks. At the end of this period the animals were divided into two groups. Each animal of group A received a single dose of 50mg. of flavone in addition to the standard diet, while all the group B animals received the standard diet only. After the first day both groups received the unsupplemented diet only. The 24hr. urine samples collected over 3 days were extracted by the standard procedure and the extracts examined by two-dimensional chromatography with solvent system *A*. Examination of chromatograms from the two groups under ultraviolet light revealed the presence on the chromatograms of group A of

strongly fluorescent compounds that were absent from those of group B (Fig. 1).

Unchanged flavone (spot 6′) was detected by spraying with 2,4-dinitrophenylhydrazine. The colour observed was dark orange on a yellow background. No colour was observed with the other fluorescent spots. The only example of a normally formed carbonyl derivative of a flavone appears to be flavone 2,4-dinitrophenylhydrazone (Baker, Harborne & Ollis, 1952). On spraying with a second spray of 5% sodium carbonate the flavone spot turned a reddish colour. The spot of unchanged flavone showed high R_F values in propan-2-ol–aq. ammonia–water and benzene–acetic acid–water (Fig. 1). Unchanged flavone was observed in the urine extracts of flavone-fed as well as flavone-injected guinea pigs.

Spot 1 was found to give reactions characteristic of a phenolic compound, namely pale blue with the ferric chloride–potassium ferrocyanide spray reagent, yellow with diazotized *p*-nitroaniline and orange–yellow with diazotized sulphanilic acid. It showed blue fluorescence in ultraviolet light becoming green in the presence of ammonia fumes.

Spot 2, which also showed blue fluorescence in ultraviolet light turning to yellow–green in the presence of ammonia, gave a very pale-blue colour with the ferric chloride–potassium ferrocyanide reagent, and on spraying with saturated aq. ammonium molybdate an immediate orange–brown colour was obtained. This has been held by Pridham (1959) to be characteristic of *o*-dihydroxyphenols. The compound was tentatively identified as 3′,4′-dihydroxyflavone by co-chromatography with an authentic specimen of this compound. Comparison of the ultraviolet spectra of the eluted compound and the standard preparation showed that these also were in agreement, showing absorption maxima at 343 and 244mμ. However, since the compound was present in trace quantities only on these chromatograms, it was not possible to obtain the dihydroxyflavone in the crystalline state.

Spot 3 gave a very pale-blue colour with the ferric chloride–potassium ferrocyanide spray reagent, a purple colour with aq. 3% ferric chloride and red–brown and orange–yellow colours with diazotized *p*-nitroaniline and diazotized sulphanilic acid respectively. By co-chromatography with an authentic specimen of salicylic acid in solvent systems *A* and *B*, it was tentatively identified as that compound. However, since it was present on the chromatograms in trace quantities only, it was not possible to characterize it further. No other phenolic metabolites of flavone were detected, nor was there any increase in the amounts of the phenolic acids normally found on the control chromatograms. The other fluorescent spots (4–8) gave no colours with these reagents.

Identification and isolation of 4′-hydroxyflavone. Co-chromatography in solvent systems *A* and *B* showed that the chromatographic behaviour of the compound corresponding to spot 1 (compound 1) was identical with that of 4′-hydroxyflavone. Both showed similar intense blue fluorescence in ultraviolet light, turning to green on exposure to ammonia, identical colour reactions with the diazotized reagents and positive reactions with the ferric chloride–potassium ferrocyanide reagent.

Six guinea pigs were taken and each was given 50mg. of flavone orally for 3 days. Their urines were pooled, hydrolysed with concentrated hydrochloric acid under reflux and extracted with ether in the usual way. Urine collection was continued for 4 consecutive days. The ether extracts were pooled, concentrated and submitted to band chromatography on Whatman no. 17 paper. The eluates of 4′-hydroxyflavone obtained were concentrated and refractionated several times on pre-washed sheets of Whatman no. 17 paper. The final eluate of the 4′-hydroxyflavone in acetone–ethanol (1:1, v/v) was concentrated to 10ml. and to it was added about 30ml. of water. Compound 1 was precipitated as white crystals. The solid was recrystallized several times and the pure crystals were dried for 6hr. under reduced pressure at 120° and over phosphorus pentoxide. The dried solid, which was in the form of white flakes, melted at 269–270°, which is the m.p. of 4′-hydroxyflavone.

The infrared spectra of both compound 1 and 4′-hydroxyflavone over the range 2·5–16μ were also identical.

Determination of the ultraviolet spectra of a pure specimen of 4′-hydroxyflavone and of the compound corresponding to spot 1 showed that both compounds possessed identical absorption maxima at 327 and 254mμ.

After alkaline degradation of compound 1 with aqueous potassium hydroxide, an ether extract of the hydrolysis products was submitted to two-dimensional chromatography in solvent system *A*. With the ferric chloride–potassium ferrocyanide reagent and the diazotized *p*-nitroaniline reagent, the presence of at least four phenolic degradation products was shown (Fig. 2). Similarly it was shown that alkaline degradation of an authentic specimen of 4′-hydroxyflavone gave the same pattern of spots on the chromatogram, and by co-chromatography the identity of the two series was established. By co-chromatography with pure standard substances the chromatographic behaviour of spots 1″ and 2″ was shown to be identical with that of salicylic acid and *p*-hydroxybenzoic acid respectively. Spot 4″, which gave a strong reaction with the ferric chloride–potassium ferrocyanide reagent, was not identified.

It was shown by co-chromatography that spot

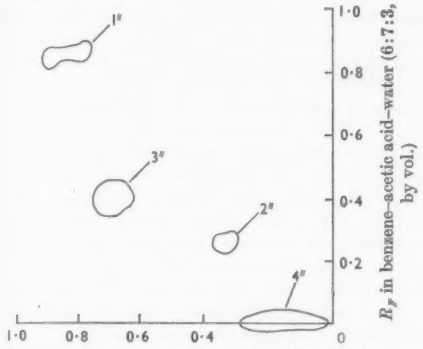

Fig. 2. Two-dimensional chromatogram of degradation products of compound 1 after treatment with 50% KOH, as described in the Experimental section. Colours indicated below were those obtained by spraying with diazotized *p*-nitroaniline. Spot 1″ (salicylic acid): red–brown. Spot 2″ (*p*-hydroxybenzoic acid): red. Spot 3″ (*p*-hydroxyacetophenone): red–brown. Spot 4″ (unidentified).

3″ possessed similar chromatographic properties to *p*-hydroxyacetophenone. The presence of both *ortho* and *para* isomers in the hydrolysate was shown by determination of their absorption spectra. The *o*-hydroxyacetophenone, which formed oily droplets that floated in the alkaline hydrolysate, was removed by physical means and washed with a small volume of water. The absorption spectra, which showed absorption maxima at 252 and 326 mμ in aqueous 0·1 N-hydrochloric acid and 0·1 N-sodium hydroxide respectively, were in agreement with those given by an authentic specimen of *o*-hydroxyacetophenone. Chromatographic separation of the other constituents of the hydrolysate followed by spectrophotometric examination of the eluates showed that the absorption maxima of the compounds believed to be *p*-hydroxyphenylacetic acid, salicylic acid and *p*-hydroxyacetophenone possessed absorption maxima at 256, 305 and 275 mμ respectively in aqueous 0·1 N-hydrochloric acid and at 282, 297 and 325 mμ respectively in aqueous 0·1 N-sodium hydroxide. These values are in agreement with those obtained with solutions of the authentic compounds.

Since it has been shown (Muller, 1915) that flavone under similar conditions of hydrolysis gives rise to salicylic acid, *o*- and *p*-hydroxyacetophenone and benzoic acid, the products reported here are those that would be expected to originate from fission of the 4′-hydroxyflavone molecule. Although spot 4″ was not identified, it was shown that the spot was present on chromatograms of the hydrolysis products of the pure 4′-hydroxyflavone as well as on those of the metabolite. On theoretical grounds, it is thought probable that the compound is the intermediate, 2,4′-dihydroxydibenzoylmethane.

The amount of 4′-hydroxyflavone excreted by flavone-fed guinea pigs was determined by the spectrophotometric method. Six animals maintained on the standard diet for a week before the experiment were each given 30 mg. of flavone. The urines, collected over 24 hr. periods, were pooled and the mean values determined over 4 days. It was shown that 622 μg., 309 μg., 15 μg. and 0 μg./guinea pig/24 hr. were excreted on the first, second, third and fourth day of the experiment respectively. These results show that excretion is maximal during the first 24 hr. after administration. The amount of 4′-hydroxyflavone formed is small in relation to the amount of flavone given.

Formation of 4′-hydroxyflavone. Since previous studies had shown that the degradation of a related substance, (+)-catechin, in the mammal was dependent on the activities of the microflora in the gut (Griffiths, 1964), consideration was given to a possible role of the intestinal bacteria in the degradation of flavone. Flavone (30 mg.) was given to each of two groups (A and B) of guinea pigs daily for 7 days. In group B only, an additional supplement of phthaloylsulphathiazole (70 mg.) and aureomycin (50 mg.) was given to each animal from the third to the seventh day. Although the antibacterial substances at these doses almost completely suppress the degradation of catechin in the rat (Griffiths, 1964), chromatograms showed no decrease of flavone metabolites in the antibiotic-treated group. Further evidence that the intestinal microflora is not of importance in the metabolism of flavone in the guinea pig was obtained by administering 30 mg. of flavone in sterile acetone intraperitoneally to each of a group of six animals while a second group of six each received 30 mg. of flavone orally. Under these conditions chromatograms prepared from the urine of the two groups were identical and it was concluded that formation of the metabolites was not dependent on the action of the intestinal microflora.

In view of the report of Winicki, Chopin, Cier & Nofre (1960) that flavone is hydroxylated by a non-enzymic system containing ascorbic acid, oxygen and ferrous chelate, consideration was also given to the possibility that hydroxylation of ingested flavone in the guinea pig might occur not in the tissues of the animal but in the digestive tract in the presence of dietary ascorbic acid, which is given in large amounts in the standard diet to compensate for the elimination of green leaf material from the diet. However, since flavone administered parenterally gives rise to the excretion of similar amounts

of the metabolites to those found after oral administration, it appears probable that hydroxylation of the flavone molecule normally occurs after absorption from the gut.

Examination of the urine of flavone-fed animals for aromatic acid metabolites. A group of six animals that had been maintained on the standard diet for 3 weeks was divided into two groups. The urine from animals of the first group, which had received a single dose of 50mg. each, was collected over three 24hr. periods and extracted with ether. Urine of the second group, which had received no flavone over this period, was treated similarly. On chromatography of the ethereal extracts in the propan-2-ol–aq. ammonia–water solvent system, followed by the spraying of duplicate chromatograms with ethanolic bromocresol green and the 8-hydroxyquinoline sulphate reagent respectively, it was shown that no additional aromatic acids were present on the chromatograms of the flavone-fed animals other than a trace amount of salicylic acid (R_F 0·90 in propan-2-ol–aq. ammonia–water). Duplicate chromatograms were also dipped in the *p*-dimethylaminobenzaldehyde reagent. No additional aroyl-glycine spots were found on the chromatograms of the urine from flavone-fed animals, but a single spot of hippuric acid, of equal size and intensity, was present on chromatograms of the experimental and control groups.

DISCUSSION

Although hydroxylation of flavone, yielding 4′-hydroxyflavone with small amounts of 3′,4′-dihydroxyflavone, has been shown to occur in the guinea pig, formation of aromatic acids from flavone occurs, if at all, to a very limited extent, as salicylic acid in trace amounts was the only compound of this type detected. In view of previous observations (Booth *et al.* 1956, 1958*a*,*b*; Griffiths, 1964) of the formation of a number of phenyl-acyl acids from ring B of a wide range of naturally occurring flavonoid molecules, it was expected that the corresponding aromatic acids, phenylpropionic acid or phenylacetic acid or their aroyl-glycine derivatives, would be formed in appreciable amounts if ring-fission of flavone occurred. The absence of these compounds suggests that flavone is more resistant to ring-fission than the naturally occurring flavonoids investigated previously (Booth *et al.* 1956, 1958*a*,*b*; Griffiths, 1964). If, however, flavonoid molecules are, as suggested by Masri, Booth & De Eds (1959), degraded from the A ring end of the molecule it would be expected that the absence of hydroxylation in this ring would render the molecule less susceptible to oxidative attack. The possibility that other hydroxyflavones may also be formed in smaller amounts cannot be ruled out, since other fluorescent spots were observed on the chromatograms but were not identified owing to the small amounts present and the absence of suitable chromatographic standards.

We are grateful to Professor T. A. Geissman, Professor T. R. Seshadri and Dr L. Jurd for their gifts of several hydroxyflavones. This investigation forms part of a Thesis to be submitted by N. P. D. to the University of Singapore in partial fulfilment of the requirements for the degree of Ph.D.

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A New Flavan-3,4-diol from *Acacia auriculiformis* by Paper Ionophoresis

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(*Received* 23 *July* 1965)

1. The heartwood of *A. auriculiformis* contains a typical mixture of analogues consisting of three isomeric flavan-3,4-diols, a dihydroflavonol, flavanone, flavonol and chalcone based on the 4′,7,8-trihydroxyl pattern. These were resolved by preparative paper chromatography and preparative paper ionophoresis. 2. Crystalline (−)-teracacidin [(2*R*,3*R*,4*R*)-4′,7,8-trihydroxy-2,3-*cis*-flavan-3,4-*cis*-diol] was obtained in high (10%) yield, and a new crystalline derivative of (−)-isoteracacidin [(−)-2,3-*cis*-3,4-*trans* isomer] was isolated. The crystalline methyl ether of a new (+)-2,3-*trans*-3,4-*cis* isomer was isolated. 3. The absolute configurations of (−)-isoteracacidin (2*R*,3*R*,4*S*) and of the (+)-2,3-*trans*-3,4-*cis* isomer (2*R*,3*S*,4*S*) were tentatively assigned on the basis of nuclear-magnetic-resonance spectroscopy, paper ionophoresis and paper-chromatographic comparison with the epimerization products of (−)-teracacidin. 4. Possible reasons for the absence of polymeric leuco-anthocyanidin tannins are discussed. 5. (±)-4′,7,8-Trihydroxydihydroflavonol and (±)-4′,7,8-trihydroxyflavanone were isolated for the first time. 6. The bark polyphenols consist mainly of polymeric leuco-delphinidins and leuco-cyanidins which redden exceptionally rapidly to light. The mechanism of this phenomenon is discussed.

The heartwood of *Acacia auriculiformis* A. Cunn. contains a mixture of phenolic components that is characterized by the prominence of three flavan-3,4-diols but absence of significant amounts of complex leuco-anthocyanidin tannins which frequently accompany them in related *Acacia* spp. This interesting observation prompted a closer examination of the heartwood components to account for a phenomenon that could be related to certain structural factors or to the absence of enzymes.

(−)-Teracacidin[(−)-4′,7,8-trihydroxy-2,3-*cis*-flavan-3,4-*cis*-diol] is shown to be the predominant component, and is accompanied in high proportion by the geometric isomers (−)-isoteracacidin (2,3-*cis*-3,4-*trans*) and a new (+)-4′,7,8-trihydroxy-2,3-*trans*-flavan-3,4-*cis*-diol. The last-named were concurrent on paper chromatograms, but their methyl ethers were elegantly resolved by paper ionophoresis on a preparative scale.

The flavan-3,4-diols are accompanied by the usual pattern (cf. Roux & Paulus, 1960, 1961*a*,*b*, 1962; Drewes & Roux, 1963) of dihydroflavonol, flavanone, flavonol and chalcone analogues.

EXPERIMENTAL AND RESULTS

Nuclear-magnetic-resonance spectra were recorded by Dr K. Pachler, Chemical-Physics Group, C.S.I.R., Pretoria, South Africa, and by Dr J. Feeney, Varians Associates, Walton-on-Thames, Surrey, on the Varian A-60 and HA-100, spectrometers with deuterochloroform as solvent and tetramethylsilane as internal standard. Band positions are on the τ scale. Coupling constants were measured with an accuracy of $\pm 0{\cdot}2$ cyc./sec.

Compounds were dried at 100° for 2hr. under vacuum before analysis and measurement of optical rotations. C,H, methoxyl and acetyl determinations were by Dr F. Pascher and E. Pascher, Bonn, Germany. All melting points are uncorrected.

Two-dimensional chromatograms were run in water-satd. butan-2-ol and then 2% (v/v) acetic acid on sheets (18¼in. × 11½in.) of Whatman no. 1 paper. Preparative paper chromatograms were on sheets (18¼in. × 22½in.) of Whatman no. 3 paper.

Origin of samples and distribution of species. The wood and bark of *A. auriculiformis* A. Cunn. *ex* Benth. were collected by Mr D. I. Nicholson from the plantations of the Forest Department, Sandakan, Sabah, Malaysia. Cross-sections were cut at 5 and 15ft. from a tree 40ft. high with 41in. girth at breast height. The specimen was just under 11 years old.

A. auriculiformis is indigenous to Thursday Island and other islands off the coast of Northern Australia and Queensland. The species is often confused with *A. aulacocarpa* A. Cunn., but they are quite distinct.

Two-dimensional paper chromatography and colour-reactions of bark and heartwood components

Bark components. The bark contains 19·0% of hot-water-soluble substances and 18·3% of cold-water-soluble substances. Of the latter 13·6% reacted as tannins and 4·7% as non-tannins to hide powder in the empirical Official Shake Method (Atkin & Thompson, 1937). Two-dimensional chromatograms of both hot- and cold-water extractives showed an intense streak extending from the origin to R_F 0·55 in the first direction (water-satd. butan-2-ol) with limited extension (R_F 0·26) in the second direction (2% acetic acid). These polymeric tannins were accompanied by two compounds in the lower-R_F region (0·33, 0·24 and 0·33, 0·39).

These phenolic compounds all gave an ochre with *bis*-diazotized benzidine (indicative of a phloroglucinol A-nucleus in flavans), and reduced instantaneously with ammoniacal $AgNO_3$ (pyrogallol or catechol B nuclei). The tannin mixture gave only delphinidin and cyanidin chlorides in high yield with the method of Pigman, Anderson, Fischer, Buchanan & Browning (1953), and the mixture probably contains leuco-delphinidins and leuco-cyanidins in both monomeric and polymeric form. The solid bark extract and leather tanned with it rapidly develop exceptional redness when exposed to sunlight.

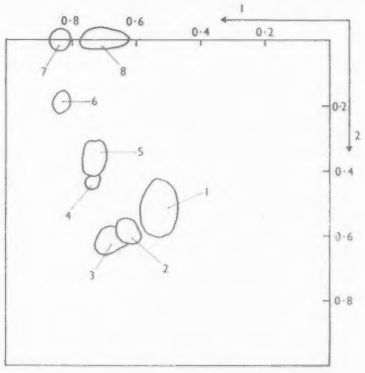


Fig. 1. Two-dimensional paper chromatography of the heartwood components of *A. auriculiformis*. Water-satd. butan-2-ol was used for the first direction and 2% (v/v) acetic acid for the second. Key to compounds: 1, (−)-teracacidin; 2, (−)-isoteracacidin; 3, (+)-4′,7,8-trihydroxy-2,3-*trans*-flavan-3,4-*cis*-diol; 4, possibly a 4′,7,8-trihydroxyflavan-3-ol, from relative position and colour reactions; 5, (±)-4′,7,8-trihydroxydihydroflavonol; 6, (±)-4′,7,8-trihydroxyflavanone; 7, 2′,3′,4′,4-tetrahydroxychalcone; 8, 4′,7,8-trihydroxyflavonol. The compounds were present in the proportions 1 (10): 2+3 (5): 4 (< 1): 5 (4): 6 (1): 7 (trace): 8 (< 1). Other spots represent unknowns.

Heartwood components. Two-dimensional paper chromatograms of the methanol-soluble components of heartwood drillings showed the presence (Fig. 1) of seven major and three minor components, when sprayed with ammoniacal $AgNO_3$. These had the following values in water-saturated butan-2-ol and 2% acetic acid respectively: (−)-tetracacidin (0·54, 0·52), (−)-isoteracacidin (0·63, 0·59), (+)-4′,7,8-trihydroxy-2,3-*trans*-flavan-3,4-*cis*-diol (0·66, 0·61), (±)-4′,7,8-trihydroxyflavanonol (0·73, 0·39), an unknown, probably 4′,7,8-trihydroxyflavan-3-ol (0·74, 0·45), (±)-4′,7,8-trihydroxyflavanone (0·83, 0·19), 2′,3′,4′,4-tetrahydroxychalcone (0·83, 0·0), 4′,7,8-trihydroxyflavonol (0·72, 0·0). Tannins, usually represented by streaks in the lower-R_F region, were either entirely absent or else present in low concentration.

All compounds showed instantaneous reduction with ammoniacal $AgNO_3$, indicating the presence of vicinal phenolic hydroxyl groups. With bis-diazotized benzidine spray all compounds gave a yellow colour. The leuco-anthocyanidins gave a dull pink with toluene-*p*-sulphonic acid spray, whereas the dihydroflavonol developed a brilliant yellow fluorescence when viewed under u.v. light after treatment with the same spray. The chalcone showed a characteristic intensification of yellow when exposed to ammonia vapour, and the flavonol gave a brilliant yellow fluorescence under u.v. light which is characteristic of 5-deoxyflavonols.

Extraction of the heartwood of A. auriculiformis. The drillings from the dry heartwood (816 g.) were extracted four times for successive periods of 1, 2, 4 and 8 days with similar volumes of methanol (total 3 l.) at room temperature. Evaporation of the combined methanolic extracts under vacuum left a brown powder (22·8 g.). This was stirred into 250 ml. of hot water (80°), and the mixture immediately filtered through a coarse filter (Whatman no. 541) leaving 1·3 g. of insoluble residue. The solubles were cooled, diluted to 650 ml. with ethanol and streaked on to 130 preparative sheets of Whatman no. 3 paper as before (Roux & Paulus, 1962). The chromatograms were developed by upward migration with 2% (v/v) acetic acid. Bands were cut corresponding to isoteracacidin and 4′,7,8-trihydroxy-2,3-*trans*-flavan-3,4-*cis*-diol (R_F 0·53), teracacidin (0·44), the dihydroflavonol (0·34), flavanone (0·17), flavonol and chalcone (0·02–0·0). These were eluted with 70% ethanol.

(−)-*Teracacidin* [(−)-4′,7,8-*trihydroxy*-2,3-cis-*flavan*-3,4-cis-*diol*]. Teracacidin (2·21 g.) crystallized readily from the eluates of band R_F 0·44 on concentration to small volume. These were obtained as fine buff needles in rosettes, m.p. 224–226° (browning with decomposition), on recrystallizing from water, $[\alpha]_D^{20}$ −73·3±0·8° (*c* 0·65 in ethanol) (Found: C, 62·2; H, 5·1. Calc. for $C_{15}H_{14}O_6$: C, 62·1; H, 4·9%). Micro-fusion with dry KOH (Roux, 1958) yielded pyrogallol and *p*-hydroxybenzoic acid.

Clark-Lewis & Dainis (1964) cite m.p. 225–226°, $[\alpha]_D$ −71°, for the same compound.

(−)-4′,7,8-*Trimethoxy*-2,3-cis-*flavan*-3,4-cis-*diol*. (−)-Teracacidin (125 mg.), in methanol, was methylated with ethereal diazomethane for 48 hr. at 0°. Clusters of fine needles (90 mg.) were obtained from methanol, m.p. 163°, $[\alpha]_D^{20}$ −71·0±0·7° (*c* 0·8 in ethanol) (Found: C, 65·3; H, 6·1. Calc. for $C_{18}H_{20}O_6$: C, 65·1; H, 6·1%). Paper ionophoresis in borate buffer under standard conditions (Drewes & Roux,

1964*b*) showed migration (+2·0cm.) characteristic of a 2,3-*cis*-3,4-*cis*-diol.

Clark-Lewis, Katekar & Mortimer (1961) cite m.p. 159°, $[\alpha]_D^{18}-65°$.

(−)-3,4-cis-*Diacetoxy*-4′,7,8-*trimethoxy*-2,3-cis-*flavan*. The trimethyl ether of (−)-teracacidin (75mg.) was acetylated with acetic anhydride–pyridine (1:1, v/v) (0·6ml.) for 24hr. at room temperature. From water a white solid (85mg.) was obtained which failed to crystallize. Reprecipitation from water gave an amorphous white solid, m.p. 63°, $[\alpha]_D^{26}-44·3\pm0·4°$ (*c* 0·6 in ethanol). Thin-layer chromatography (Kieselgel G, ¼mm. thick) with benzene–acetone (19:1, v/v) as irrigant showed only one discrete spot, R_F 0·49 (Found: C, 63·6; H, 5·6. $C_{22}H_{24}O_8$ requires: C, 63·5; H, 5·8%).

The nuclear-magnetic-resonance spectrum of the compound (Table 1) in comparison with spectra of the four possible geometrical arrangements of related flavan-3,4-diols (Drewes & Roux, 1964*a*; Saayman & Roux, 1965) showed it to be the pure 2,3-*cis*-3,4-*cis* form.

Separation of overlapping flavan-3,4-diol components by successive thin-layer chromatography and paper ionophoresis of their methyl ethers

From the bands of R_F 0·53 a brown powder (1·28g.) was obtained on elution with aq. 70% (v/v) ethanol. The product in methanol was methylated for 48hr. at −10°. The mixture was reduced in volume (1ml.) and applied as a narrow (0·5cm.) band to 21 thin-layer chromatographic plates (20cm.×20cm.) previously coated with Kieselgel G (Merck) 1mm. thick. The chromatograms were developed in ethyl acetate–chloroform (7:3, v/v) to the tops of the plates (1·5hr.). A band (R_F 0·60) giving an orange–red with H_2SO_4–aq. 40% formaldehyde (20:1, v/v) was scraped off, eluted with acetone and concentrated to dryness at 50° (water bath) to give a white residue (465mg.). This process removed brown condensed material resulting from methylation which remained on the origin and also the 4-ethyl or 4-methyl ethers which had high mobility (approx. 0·90). Examination of the residue by paper ionophoresis in borate buffer under standard conditions (Drewes & Roux, 1964*b*) indicated that it consisted of a mixture of 2,3-*cis*-3,4-*trans* (−2·1cm. migration) and 2,3-*trans*-3,4-*cis* isomers (+3·1cm.), the former predominating.

The mixture of methylated isomeric flavan-3,4-diols (30–35mg.) in ethanol (0·5ml.) was applied as a streak on a wet Whatman no. 3 (18½in.×20½in.) sheet, previously soaked in sodium borate–boric acid buffer (pH 8·8) (Cooper & Roux, 1965). A current of 0·33mA/cm. width of paper at 150–170v was applied for 21hr. On an average, the band of the 2,3-*trans*-3,4-*cis* isomer was located (toluene-*p*-sulphonic acid spray) at +8·5cm., and the 2,3-*cis*-3,4-*trans* band at −4·0cm. The bands were cut and eluted with aq. 50% (v/v) ethanol. The combined eluates from each band from 15 successive ionophoretic runs were concentrated under reduced pressure to remove all ethanol, acidified with acetic acid and the aqueous solution was extracted exhaustively with ethyl acetate. The combined extracts of each were washed with water and concentrated to dryness at 50° (vacuum). The residues of each isomer were again subjected to preparative thin-layer chromatography under the above conditions (three plates each).

Table 1. *Nuclear-magnetic-resonance spectra of isomeric 3,4-diacetoxy-4′,7,8-trimethoxyflavans and dihydroflavonol analogue*

The solvent used was deuterochloroform, with tetramethylsilane as internal standard.

Chemical shifts: τ values (p.p.m.)

	Me (acetyl)		Me (methoxyl)			H: Heterocyclic			H: Benzenoid			
	3-	4-	7-	4′-	8-	2-	3-	4-	5-	6-	3′-+5′-	2′-+6′-
(+)-2,3-*trans*-3,4-*cis* (III)†	8·21	7·93	6·25	6·24	6·20	4·72	4·56	3·83	3·00	3·41	3·09	2·61
(−)-2,3-*cis*-3,4-*trans* (II)‡	8·13	7·88	6·19	6·11	6·09	4·68	4·77	4·12	2·97	3·41	3·09	2·56
(−)-2,3-*cis*-3,4-*cis* (I)‡	8·11	7·92	6·20	6·13	6·11	4·67	4·38	3·68	3·09	3·41	3·11	2·57
			Phenolic acetyls									
(±)-2,3-*trans*-Dihydroflavonol (V)‡	8·00	—	7·81	7·72		4·63	4·29	—	2·24	3·13	2·93	2·59

Spin–spin coupling constants for 2-, 3- and 4-protons (cyc./sec.)*

	$J_{2,3}$	$J_{3,4}$
(+)-2,3-*trans*-3,4-*cis*	10.2	3·1
(−)-2,3-*cis*-3,4-*trans*	1·5	2·9
(−)-2,3-*cis*-3,4-*cis*	~1·0	4·0
(±)-2,3-*trans*-Dihydroflavonol	11·9	—

* $J_{5,6}$, $J_{2',3'}$ and $J_{5',6'}$ approximate to 8·9–9·0 cyc./sec. for all compounds.
† Measured on the Varian HA-100 spectrometer.
‡ Measured on the Varian A-60 spectrometer.

Derivatives of the (+)-2,3-trans-3,4-cis-diastereoisomer of teracacidin

(+)-4′,7,8-*Trimethoxy*-2,3-trans-*flavan*-3,4-cis-*diol*. From the 2,3-*trans*-3,4-*cis* fraction in absolute ethanol, feathery needles in rosettes (30 mg.), m.p. 168–170°, were obtained. Paper ionophoresis indicated a residual trace of the 2,3-*cis*-3,4-*trans* isomer, but on recrystallization from ethanol this was entirely removed, and the product (24 mg.) had m.p. 179°, $[\alpha]_D^{20}+7{\cdot}0\pm1{\cdot}6°$ (*c* 0·3 in ethanol) (Found: C, 64·9; H, 6·0; OMe, 27·7. $C_{18}H_{20}O_6$ requires C, 65·1; H, 6·1; OMe, 28·0%).

The compound had a mobility (+3·1 cm.) in sodium borate buffer under standard conditions characteristic of methylated 2,3-*trans*-3,4-*cis*-diols (Drewes & Roux, 1964*b*, 1965*a*; Saayman & Roux, 1965). Similarly, its mobility in sodium borate–boric acid buffer (pH 8·8) (Cooper & Roux, 1965) on Schleicher and Schull no. 2043 (4 cm. × 41 cm.) paper strips was +6·7 cm. at 0·31 mA/cm. width, and 150 v for 15 hr.

(+)-3,4-cis-*Diacetoxy*-4′,7,8-*trimethoxy*-2,3-trans-*flavan*. The trimethyl ether (15 mg.) was acetylated with acetic anhydride–pyridine (1:1, v/v) (0·1 ml.) for 20 hr. at room temperature. From water a white amorphous solid (17·9 mg.) melting slowly from 52° onwards was obtained (Found: C, 64·4; H, 6·2. $C_{22}H_{24}O_8$ requires C, 63·5; H, 5·8%). The compound failed to crystallize from common solvents.

The nuclear-magnetic-resonance spectrum of the 2,3-*trans*-3,4-*cis*-diacetate (Table 1), when compared with previous work on related flavan-3,4-diols of similar relative configuration (Drewes & Roux, 1964*a*, 1965*a*; Clark-Lewis, Jackman & Williams, 1962; Saayman & Roux, 1965), confirmed the 2,3-*trans*-3,4-*cis* assignment.

New derivatives of isoteracacidin

(−)-4′,7,8-*Trimethoxy*-2,3-cis-*flavan*-3,4-trans-*diol*. After separation by preparative paper ionophoresis and thin-layer chromatography the 2,3-*cis*-3,4-*trans* fraction from methylation with diazomethane yielded a white solid (180 mg.) which did not crystallize, m.p. 68–78°, $[\alpha]_D^{20}-40{\cdot}3\pm0{\cdot}9°$ (*c* 0·7 in ethanol) (Found: C, 65·2; H, 6·2; OMe, 27·7. $C_{18}H_{20}O_6$ requires: C, 65·1; H, 6·1; OMe, 28·0%).

The compound had the mobilities −1·7 cm. in sodium borate buffer (Drewes & Roux, 1964*b*), and −2·1 cm. in sodium borate–boric acid (Cooper & Roux, 1965).

(−)-3,4-trans-*Diacetoxy*-4′,7,8-*trimethoxy*-2,3-cis-*flavan*. The amorphous trimethyl ether (52 mg.) was acetylated with acetic anhydride–pyridine as above, giving a white solid (58 mg.) from water. This crystallized readily from ethanol, m.p. 149–150° (Found: C, 64·0; H, 6·0; OMe, 22·0; $CO{\cdot}CH_3$, 21·5. $C_{22}H_{24}O_8$ requires C, 63·5; H, 5·8; OMe, 22·4; $CO{\cdot}CH_3$, 20·7%).

The nuclear-magnetic-resonance spectrum of the diacetate (Table 1) was similar to those of flavan-3,4-diols of the same 2,3-*cis*-3,4-*trans* configuration (see above). The presence of a trace of the corresponding 2,3-*trans*-3,4-*trans* isomer was indicated by a weak acetyl signal at τ 8·02 p.p.m.

(±)-4′,7,8-*Trihydroxy*-2,3-trans-*dihydroflavonol*. The compound (157 mg.) crystallized from aqueous solution of the eluates of band R_F 0·34 after concentration to small (2 ml.) volume. Recrystallization from water gave colourless needles, m.p. 220–221° with browning, $[\alpha]_D^{20}+3{\cdot}0\pm0{\cdot}6°$ (*c* 0·7 in ethanol) (Found: C, 62·0; H, 4·2. $C_{15}H_{12}O_6$ requires C, 62·5; H, 4·2%). This low rotation suggested that the compound was racemic, and this was confirmed by hydrogenation over platinum oxide (Adams catalyst) which afforded enantiomeric 4′,7,8-trihydroxy-2,3-*trans*-flavan-3,4-*trans*-diols [cf. Drewes & Roux (1964*a*), Roux & Paulus (1960) and Freudenberg & Weinges (1959) for the analagous hydrogenations of (+)- and (−)-fustins] as shown by two-dimensional paper chromatography. The dihydroflavonol gives a dark purple with Mg–HCl, and affords the flavonol analogue on heating with mineral acid (chromatographic evidence).

(±)-3,4′,7,8-*Tetracetoxy*-2,3-trans-*dihydroflavonol*. The dihydroflavonol (43 mg.) was acetylated at room temperature with 0·4 ml. of acetic anhydride and 0·2 ml. of pyridine. A white solid (50 mg.) was recovered from water. This crystallized from ethanol in clusters of prisms (39 mg.), m.p. 159–160° (Found: C, 60·0; H, 4·8. $C_{23}H_{20}O_{10}$ requires C, 60·5; H, 4·4%).

The 2,3-*trans* configuration of the racemate is evident from the large coupling constants of the 2- and 3- protons [$J_{2,3}$ 11·9 cyc./sec. (Table 1)].

(±)-4′,7,8-*Trihydroxyflavanone*. After the formation of a brown sludge, the compound crystallized from the aqueous concentrate of the eluates of band R_F 0·17. Recrystallization from water gave needles (21 mg.), m.p. 104–105°. The compound gave a deep blue with Zn–HCl reagent, typical of flavanones.

(±)-4′,7,8-*Triacetoxyflavanone*. The flavanone (16 mg.) was acetylated as above. Long needles (15 mg.), m.p. 165–166°, were obtained from ethanol, $[\alpha]_D^{20}-2{\cdot}9\pm0{\cdot}8°$ (*c* 0·3 in ethanol) (Found: C, 63·0; H, 4·6. $C_{21}H_{18}O_8$ requires C, 63·3; H, 4·6%).

4′,7,8-*Trihydroxyflavonol*. The eluates of band R_F 0·02 were concentrated and dissolved in methanol (80 ml.). The methanolic solution was streaked on to 16 sheets of Whatman no. 3 paper, and the chromatograms were developed downward with aq. acetic acid (13:30, v/v). Four bands were located as follows: R_F 0·48, yellow fluorescent under u.v. light; 0·57, yellow, intensified when exposed to ammonia vapour; 0·65, bright-yellow fluorescent under u.v. light, and 0·74, dark under u.v. light, becoming yellow when exposed to ammonia vapour.

The band R_F 0·48 gave the crude flavonol (200 mg.) on stripping. After recrystallizing twice from ethanol–water (3:1, v/v) pale-yellow needles were obtained, m.p. 310° (decomp.) (Found: C, 63·0; H, 4·1. Calc. for $C_{15}H_{10}O_6$: C, 62·9; H, 3·5%).

Clark-Lewis & Dainis (1964) found m.p. 310–319° for the same compound from *A. sparsiflora*, whereas Kostanecki & Schreiber (1905) found m.p. 319° for the synthetic compound.

3,4′,7,8-*Tetra-acetoxyflavone*. The flavonol (20 mg.) was acetylated as before, yielding buff needles (18 mg.) from ethanol, m.p. 180–181° after sintering at 165–170° (Found: C, 61·0; H, 4·4. Calc. for $C_{23}H_{18}O_{10}$: C, 60·8; H, 4·0%). Kostanecki & Schreiber (1905) found m.p. 175° for the synthetic compound, and Clark-Lewis & Dainis (1964) found 170–175° for the same compound derived from *A. sparsiflora*.

2′,3′,4′,4,-*Tetrahydroxychalcone*. The yellow band R_F 0·57 from the above separation intensified to a deep yellow when exposed to ammonia vapour, a reaction typical of chalcones. The solids obtained from eluates of the band refused to crystallize from ethanol–water, and after further purification by chromatography in water-

saturated butan-2-ol had $\lambda_{max.}$ 284 and 357 mμ. The quantity available was too small for further work.

Examination of related Acacia *spp. for the presence of* 2,3-trans-3,4-cis-*flavan*-3,4-*diols*

The heartwood extract of *A. intertexta* Sieb. was found to contain (−)-melacacidin [(−)-3′,4′,7,8-tetrahydroxy-2,3-*cis*-flavan-3,4-*cis*-diol] by two-dimensional paper chromatography. Its geometrical isomers (see below), as well as its dihydroflavonol, flavanone, flavonol and chalcone analogues were also present. Bands corresponding to isomelacacidin were cut from preparative chromatograms run in 2% acetic acid. Solids obtained from the eluates of these bands were methylated, and the product was examined by paper ionophoresis under standard conditions (Drewes & Roux, 1964*b*). The presence of 2,3-*cis*-3,4-*trans* and 2,3-*trans*-3,4-*cis* diastereoisomers of (−)-melacacidin was established in this way. The flavonoid mixture from the heartwood of *A. intertexta* is an exact parallel to the pattern present in *A. auriculiformis* except for its added degree of (3′-) hydroxylation.

The 2,3-*trans*-3,4-*cis* diastereoisomer of (−)-melacacidin and isomelacacidin, was shown to be absent from *A. melanoxylon* R. Br. heartwood by similar means.

Epimerization of (−)-*teracacidin*

Teracacidin (5 mg.) in water (5 ml.) was autoclaved for 2 hr. at 15 lb./in.^{2} (Drewes & Roux, 1964*d*, 1965*a*) and the product examined by two-dimensional chromatography. Only two reducing areas were visible with ammoniacal $AgNO_3$ spray, the starting material (R_F 0·54, 0·52) and compounds of higher mobility (0·68, 0·59).

The same product, examined by two-dimensional chromatography with water-satd. phenol (40 cm. migration) and then 2% (v/v) acetic acid (approx. 50 cm. migration), showed the presence of four diastereoisomers: *cis–cis* (0·60, 0·44), *trans–trans* (0·65, 0·49), *cis–trans* (0·62, 0·54) and *trans–cis* (0·68, 0·54). The *cis–trans* and *trans–cis* isomers were recognized by comparison with the corresponding natural isomers from *A. auriculiformis* and the *trans–trans* isomer with the catalytic (Adams catalyst) hydrogenation products of racemic 4′,7,8-trihydroxy-2,3-*trans*-dihydroflavonol from the same source. The various isomers were present in the following proportions after 2 hr.: *cis–cis* (55%), *cis–trans* (12%), *trans–trans* (8%) and *trans–cis* (25%) (cf. Drewes & Roux, 1965*c*).

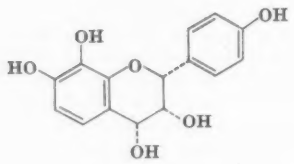
DISCUSSION

The heartwood of *A. auriculiformis* contains flavan-3,4-diols, dihydroflavonol, flavanone, flavonol and chalcone analogues based on the 4′,7,8-trisubstituted pattern of phenolic hydroxylation. This represents the first established occurrence of a wide group of analogues with this pattern, but the association parallels others with different patterns among the Leguminosae, e.g. *A. mearnsii* bark (3′,4′,5′,7-tetrahydroxy and 3′,4′,7-trihydroxy) and heartwood (3′,4′,7-trihydroxy) (Roux & Paulus, 1960, 1961*a*,*b*; Drewes & Roux, 1963) and the heartwoods of *A. melanoxylon* (3′,4′,7,8-tetrahydroxy) (Clark-Lewis & Mortimer, 1960), *A. dealbata*, *A. decurrens*, *A. pycnantha* (3′,4′,7-trihydroxy) (Roux, Maihs & Paulus, 1961) and *Robinia pseudacacia* (3′,4′,7-trihydroxy and 3′,4′,5′,7-tetrahydroxy) (Roux & Paulus, 1962). The characteristic distribution pattern of these classes of flavonoids (cf. Fig. 1) on two-dimensional chromatograms may now be readily recognized.

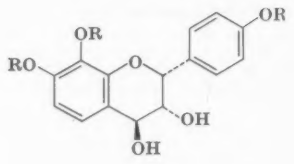
However, the polyphenolic mixture from *A. auriculiformis* differs from those above, through the presence of no less than three diastereoisomeric flavan-3,4-diols, representing (−)-2,3-*cis*-3,4-*cis*, (−)-2,3-*cis*-3,4-*trans* and (+)-2,3-*trans*-3,4-*cis* configurations. Of these (−)-teracacidin (I) (*cis–cis*) is readily separated by preparative paper chromatography, but the remaining isomers overlap and may be separated after methylation by paper ionophoresis on a preparative scale. Thus isolation of the crystalline methyl ether of the (+)-*trans–cis* isomer (III, R = CH_3) represents the first conclusive evidence of the natural presence of a flavan-3,4-diol of this configuration (III, R = H) (Drewes & Roux, 1965*d*), although a leuco-fisetinidin (3′,4′,7-trihydroxyflavan-3,4-diol) and an isomer of guibourtacacidin (4′,7-dihydroxyflavan-3,4-diol) of the same configuration was previously shown to be present in *Guibourtia coleosperma* (Drewes & Roux, 1964*e*, 1965*b*; Saayman & Roux, 1965) by paper chromatography and ionophoretic methods, and a similar isomer of (−)-melacacidin (3′,4′,7,8-tetrahydroxyflavan-3,4-diol) apparently exists in *A. intertexta*, but is absent from *A. melanoxylon*.

A crystalline trimethyl ether diacetate of (−)-isoteracacidin (2,3-*cis*-3,4-*trans* isomer) (II, R = H) was prepared for the first time, thus unambiguously demonstrating its presence in the flavan-3,4-diol mixture. (−)-Teracacidin (2,3-*cis*-3,4-*cis*) was readily obtained from *A. auriculiformis* in crystalline form, as opposed to difficulties experienced in its previous isolation in amorphous form from *A. orites* (formerly identified in error as *A. intertexta*) (Clark-Lewis *et al.* 1961; Clark-Lewis & Dainis, 1964). This compound was crystallized only recently by Clark-Lewis & Dainis (1964) from *A. sparsiflora*, but not without emphasis on the difficulties experienced. *A. auriculiformis* therefore constitutes an exceptionally rich (10% yield on extract weight) source of crystalline (−)-teracacidin (I), whereas the mixture of 2,3-*trans*-3,4-*cis* (III, R = H) and 2,3-*cis*-3,4-*trans* (II, R = H) isomers were obtained in lower (5%) yield in the crude form. These flavan-3,4-diols predominate amongst the flavonoid components.

The relative configurations of the flavan-3,4-diols were confirmed (teracacidin) and assigned (isoteracacidin and *trans–cis* isomer) by comparison of their ionophoretic mobilities and nuclear-magnetic-resonance spectra (spin–spin splitting patterns of



(I)



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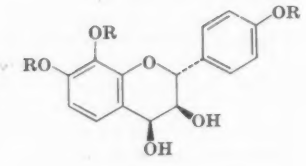

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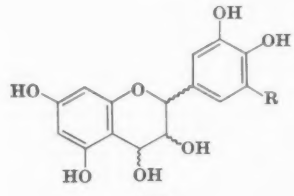

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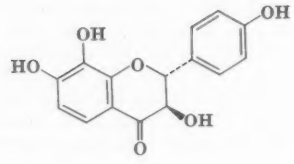

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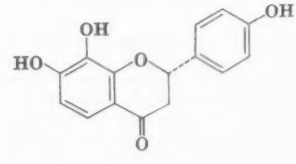

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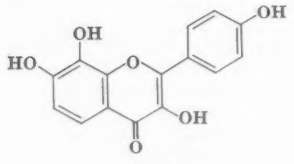

(VII)

3-, 4- and 5-protons) (Table 1) with those of other flavan-3,4-diols of similar geometrical arrangements (Clark-Lewis *et al.* 1962; Drewes & Roux, 1964*a*,*b*, 1965*a*; Saayman & Roux, 1965).

Presuming a half-chair conformation for the heterocyclic ring and a 2(*eq*) arrangement for the 2-phenyl group, the coupling constants of the 2(*ax*)-, 3(*eq*)- and 3(*eq*)-,4(*ax*)- protons of 2,3-*cis*-3,4-*cis*-(−)-teracacidin ($J_{2,3} \sim 1{\cdot}0$cyc./sec. and $J_{3,4}$ 4·0cyc./sec.) correlate well with their dihedral angles of approx. 70° and 45°, as observed from Dreiding models, on the basis of the Karplus equation (Conroy, 1959). Similar correlations were possible for 2,3-*cis*-3,4-*trans*- (−)-isoteracacidin ($J_{2,3}$ 1·5cyc./sec. and $J_{3,4}$ 2·9cyc./sec.) with dihedral angles of approx. 70° and 60° for a 2(*ax*),3(*eq*),4(*eq*) arrangement of protons and for the (+)-2,3-*trans*-3,4-*cis* isomer ($J_{2,3}$ 10·0cyc./sec. and $J_{3,4}$ 3·4cyc./sec.) with dihedral angles approx. 170° and 50° for a 2(*ax*),3(*ax*),4(*eq*) arrangement of protons.

The absolute configuration of (−)-teracacidin (2*R*,3*R*,4*R*) (I) is known (Clark-Lewis & Katekar, 1962), and that of (−)-isoteracacidin (2*R*,3*R*,4*S*) (II, R = H) is now confirmed from its relative configuration, and from its formation by selective epimerization of (−)-teracacidin at C-4 (Clark-Lewis *et al.* 1961). The methyl ether of the new 2,3-*trans*-3,4-*cis* isomer has a (+)-rotation, and by analogy with the epimerization products of

(+)-mollisacacidin (Drewes & Roux, 1964*d*, 1965*a*), most likely represents the 2*R* form and therefore the 2*R*,3*S*,4*S* (III, R = H) configuration. A leuco-fisetinidin of similar configuration (paper-chromatographic evidence) is present in *G. coleosperma* (Drewes & Roux, 1964*e*, 1965*b*).

The 4′,7,8-trihydroxyl substitution in derivatives of the isomeric flavan-3,4-diols and their dihydroflavonol analogue is confirmed from their nuclear-magnetic-resonance spectra by analysis of the spin–spin splitting pattern of benzenoid protons. Thus the A_2B_2 system of the B-ring results in the typical overlap of equivalent protons (2′,6′ and 3′,5′) to give doublets which show mainly strong *ortho*-coupling ($J_{2',3'} = J_{5',6'} \approx 8{\cdot}9$–$9{\cdot}0$ cyc./sec.), whereas the 5- and 6-protons form an AB system which appear as *ortho*-coupled doublets ($J_{5,6}$ 8·9–9·0 cyc./sec.) unsplit by further *meta*- or *para*- coupling. Notable is the strong deshielding of the 5-proton in *peri*-position to the 4-carbonyl in the dihydroflavonol (cf. Table 1), as well as the paramagnetic shift of the 5-proton in (−)-isoteracacidin (4-*axial* acetoxy group) compared with (−)-teracacidin (4-*equatorial* acetoxy group) (Δ_τ 0·10 p.p.m.). This effect has been noticed in all previous work (Drewes & Roux, 1964*a*, 1965*a*; Saayman & Roux, 1965) and may be correlated with some factor of their stereochemistry at C-4.

The dihydroflavonol (V) and flavanone (VI) analogues of (−)-teracacidin have now been isolated for the first time. Both compounds are partially racemized, the (+)- and (−)-forms respectively predominating in accordance with their usual occurrence in Nature. The large coupling constant ($J_{2,3}$ 11·9 cyc./sec.) of the 4′,7,8-trihydroxydihydroflavonol indicates a diaxial arrangement of the 2- and 3-protons with a dihedral angle approaching 180° (Conroy, 1959), and therefore a 2,3-*trans* arrangement of substituents. No exception has yet been found to the 2,3-*trans* arrangement of natural dihydroflavonols, and in *A. auriculiformis* heartwood this contrasts with the predominantly 2,3-*cis* arrangement of the associated flavan-3,4-diols [(−)-teracacidin, isoteracacidin]. The flavonol (VII) analogue has been isolated from natural sources for the second time only, having recently been obtained from *A. sparsiflora* (Clark-Lewis & Dainis, 1964) in association with (−)-teracacidin and isoteracacidin.

Considering the predominance of the 4′,7,8-trihydroxy-3,4-diols in *A. auriculiformis*, the exceptional absence of tannins (related flavan-3,4-diol polymers) in the heartwood is of interest, since it contrasts with the predominance of tannins based on other phenolic patterns in *G. coleosperma* and also with their prominence in *A. mearnsii* and closely related species, and in *R. pseudacacia*. However, the presence of tannins, although in low relative proportion in the heartwoods of *A. melanoxylon* and *A. intertexta* that contain the related (same pyrogallol-derived *A. nucleus*) 3′,4′,7,8-tetrahydroxyflavan-3,4-diols [(−)-melacacidin and isomelacacidin], suggests that factors other than the reactivity of the flavan-3,4-diol nucleus (e.g. blocking of the reactive 8-position by hydroxylation, or affecting the reactivity of the benzylic 4-hydroxyl) might be responsible for the lack of biochemical condensation of the flavan-3,4-diols in *A. auriculiformis*. The absence of tannins should, perhaps, rather be sought in the absence in the wood of those enzymic systems which promote condensation or polymerization.

The bark of *A. auriculiformis* contains a preponderance of polymeric leuco-delphinidin (3′,4′,5′,5,7-pentahydroxyl pattern) (IV, R = OH) and leuco-cyanidin (3′,4′,5,7-tetrahydroxyl) (IV, R = H) tannins associated with traces of the corresponding flavan-3,4-diols. These differ completely from phenolic heartwood components which are based on a single and different (4′,7,8-trihydroxyl) pattern. The leuco-delphinidin and leuco-cyanidin bark tannins redden exceptionally rapidly when exposed to sunlight in the solid form, in solution, or when combined with collagen in leather. The exceptional reddening is attributed to the greater electron release from the 7-hydroxyl groups of 5,7-disubstituted tannins, than from 7,8-dihydroxyl (melacacidin and teracacidin isomers and polymers) or 7-hydroxyl (leuco-robinetinidin and leuco-fisetinidin isomers and polymers) tannins (cf. Hammet δ-constants; Clark-Lewis, 1962). The electron effect of the 5-hydroxyl reinforces that of the 7-hydroxyl leading to enhanced reactivity of the benzylic 4-hydroxyl and the facile formation of a chromophore (7-keto anhydrobase of the flavylium structure) (Drewes & Roux, 1964*c*; Roux & Drewes, 1965).

Epimerization of (−)-teracacidin by autoclaving (cf. Drewes & Roux, 1965*a*) gives rise to three new geometrical isomers as with (−)-leuco-fisetinidin (Drewes & Roux, 1964*e*, 1965*b*). These may be resolved with water-saturated phenol and then 2% acetic acid on paper chromatograms (cf. Drewes & Roux, 1965*c*). The natural (−)-isoteracacidin from *A. auriculiformis* was found to be concurrent with the (−)-isoteracacidin (2,3-*cis*-3,4-*trans*) epimerization product, whereas the natural (+)-2,3-*trans*-3,4-*cis* isomer migrated to a position slightly in advance of the corresponding (−)-epimerization product, as expected. This chromatographic evidence confirms the above assignments.

This work is jointly supported by grants from the African Territories Wattle Industry Fund and the South African Council for Scientific and Industrial Research. Thanks are due to Mr D. I. Nicholson, Forest Department, Sandakan, Sabah, Malaysia, for the samples of *A. auriculiformis* and

to Dr Mary D. Tindale, Royal Botanic Gardens, Sydney, New South Wales, Australia, for a sample of *A. intertexta*.

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The Kinetics of Inhibition of Erythrocyte Cholinesterase by Monomethylcarbamates

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(*Received* 27 *July* 1965)

1. The kinetics of the interaction of erythrocyte cholinesterase with 1-naphthyl *N*-methylcarbamate, 2-isopropoxyphenyl *N*-methylcarbamate and phenyl *N*-methylcarbamate were studied. Rate constants for inhibition and rate constants for spontaneous reactivation were determined. The calculated rate constants for spontaneous reactivation agreed well with those obtained experimentally. 2. The degree of inhibition obtained after preincubation of enzyme and inhibitor was found to be independent of both the substrate concentration and the dilution of the inhibited enzyme. 3. The reaction between the enzyme and the inhibitor was consistent with carbamates being regarded as poor substrates of cholinesterases. There was no evidence for the formation of a reversible complex between the enzyme and the carbamate.

Carbamates are known as inhibitors of cholinesterases and the mechanism of inhibition is considered analogous to the mechanism of inhibition of cholinesterase by organophosphorus compounds (Wilson, Hatch & Ginsburg, 1960). In distinction to the phosphorylated enzyme, carbamoylated cholinesterases reactivated more rapidly. The rapid spontaneous reactivation creates difficulties in determining the degree of inhibition of the enzyme in the presence of carbamates (Witter, 1963). Winteringham & Disney (1964) reported that the evaluation of cholinesterase inhibition by carbamates depends on the concentration of the substrate present during the measurement of enzyme activity, as well as on the degree of dilution of the inhibited enzyme. They calculated the dissociation constants between 2-isopropoxyphenyl *N*-methylcarbamate and housefly head or human whole blood cholinesterase. On the other hand, Wilson *et al.* (1960) obtained results which allowed them to omit the reversible complex in presenting the overall reaction scheme of the inhibitory process.

In this paper a kinetic study of the inhibition of erythrocyte cholinesterase by some monomethylcarbamates is presented. Both the inhibition and the rate of spontaneous reactivation of the inhibited enzyme in the presence of substrate have been determined. The effect of dilution of the inhibited enzyme preparation and the effect of substrate concentration on the enzyme inhibition have been studied.

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MATERIALS AND METHODS

The enzyme preparation used was purified bovine erythrocyte cholinesterase (Winthrop, New York, N.Y., U.S.A.). Stock solutions (5 mg./ml.) were prepared in 1% gelatin and kept at 4°.

The substrates, acetylcholine chloride, acetylcholine perchlorate and acetylthiocholine iodide, were obtained from Pliva, Zagreb, Yugoslavia, British Drug Houses Ltd., Poole, Dorset, and Calbiochem, Los Angeles, Calif., U.S.A., respectively. The 5,5′-dithiobis-(2-nitrobenzoic acid) reagent was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.

Three monomethylcarbamates were used as inhibitors: 1-naphthyl *N*-methylcarbamate, 2-isopropoxyphenyl *N*-methylcarbamate and phenyl *N*-methylcarbamate. The compounds were provided as technical products (95–99% purity) from the World Health Organisation, Geneva, Switzerland, and were several times recrystallized from ethanol and water. Stock solutions (0·05 M) prepared in dimethylformamide were kept at 4°, and were suitably diluted with buffer or 0·9% NaCl soln. immediately before use.

Two methods were chosen for measuring cholinesterase activity in the presence of carbamates: the titrimetric method of Jørgenson (1959) and the spectrophotometric method of Ellman, Courtney, Andres & Featherstone (1961). These methods were used because the activities of enzyme could be obtained within a short time (several minutes) of assay, and the activities could be continually measured from 20 to 30 sec. after addition of substrate. At low substrate concentrations (below 1·0 mM) the spectrophotometric method has advantages because smaller enzyme concentrations are required than in the titrimetric procedure. To follow the rate of reactivation the standard manometric method was used (Aldridge, 1950).

Titrimetric determination of enzyme inhibition. The

enzyme was incubated with a given concentration of inhibitor at 25° in 0·9% NaCl soln. at an adjusted pH 7·4. At suitable time-intervals samples (10·0 ml.) were withdrawn for assay. After adding the substrate (2·0 ml. of a given concentration of acetylcholine perchlorate) the activity of enzyme was determined by an automatic titration assembly (Radiometer, Copenhagen, Denmark). The acetic acid liberated was titrated with 0·02 N-NaOH for 2–5 min. During this period, for all concentrations of acetylcholine used the amount of alkali used was linear with time both for uninhibited and inhibited enzyme samples. The final concentration of enzyme preparation during assay was 0·15 mg./ml.

Spectrophotometric determination of enzyme inhibition. The enzyme was incubated with a given concentration of inhibitor at 20° in 0·1 M-phosphate buffer, pH 7·4. At suitable time-intervals samples (3·0 ml.) were withdrawn and added to 100 μl. of dithiobisnitrobenzoate reagent plus 50 μl. of substrate solution (acetylthiocholine iodide of a given concentration). The reaction mixture was immediately transferred to a 1·0 cm. photocell and the extinction was read, against a blank which did not contain the enzyme, on a Beckman spectrophotometer (model DU) at 412 mμ. Readings were done at 30 sec. intervals for 2–5 min., the first reading being taken 30 sec. after the enzyme had been added to the reagents. During the assay the extinction was linear with time for both inhibited and uninhibited enzyme samples. The final concentration of enzyme during assay was 0·015 mg./ml.

In experiments where the effect of dilution was studied samples (x ml.) were taken from the reaction mixture of enzyme and inhibitor, and added to $3-x$ ml. of phosphate buffer plus 100 μl. of dithiobisnitrobenzoate, and 50 μl. of substrate solution, giving a total volume of 3·150 ml.

Measurement of spontaneous reactivation. The enzyme was incubated with a given concentration of inhibitor in the main compartment of the Warburg flask in 1·8 ml. of bicarbonate buffer, in an atmosphere of CO_2+N_2 (5:95) at pH 7·4 and 25°. After a suitable time-interval 0·02 ml. of substrate (acetylcholine chloride of a given concentration) was added, and the CO_2 output was followed for 1–2 hr. Readings of the CO_2 output were stopped when the amount was reached which in the control flask was no longer linear with time (about 250 μl.).

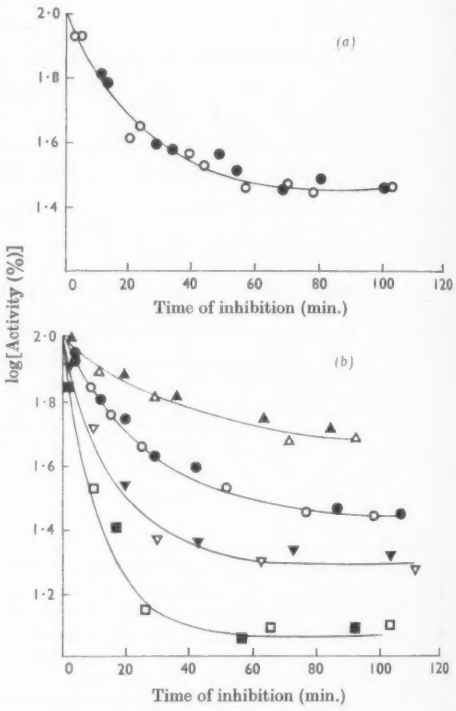

Fig. 1. Degree of inhibition of erythrocyte cholinesterase by 2-isopropoxyphenyl *N*-methylcarbamate measured at different substrate concentrations by (*a*) the titrimetric and (*b*) the spectrophotometric method. (*a*) Substrate concn.: 1·5 mM (○) and 0·15 M (●); inhibitor concn.: 1·0 μM. (*b*) Substrate concn.: 0·1 mM (△, ○, ▽, □) and 1·0 mM (▲, ●, ▼, ■); inhibitor concn. (μM): 0·5 (△, ▲), 1·0 (○, ●), 2·0 (▽, ▼) and 4·0 (□, ■).

RESULTS

Degree of inhibition as measured at different substrate concentrations. After the enzyme had been incubated with 2-isopropoxyphenyl *N*-methylcarbamate for different times the activity of cholinesterase was measured either titrimetrically at 0·15 M and 1·5 mM substrate concentration, or by the spectrophotometric method at a substrate concentration of 1·0 mM and 0·1 mM. As shown in Fig. 1 the degree of inhibition was not influenced by the concentration of the substrate used.

Similar results were obtained when the inhibition of enzyme by 1-naphthyl *N*-methylcarbamate was estimated titrimetrically at 0·15 M and 1·5 mM substrate concentration.

Degree of inhibition as measured after dilution of the inhibited enzyme preparation. The enzyme was incubated with a given concentration of 2-isopropoxyphenyl *N*-methylcarbamate for different times, and the activity of cholinesterase was measured spectrophotometrically at a substrate concentration 1·0 mM. Immediately before assay different dilutions of the inhibited enzyme were prepared. The same degree of inhibition was obtained whether the inhibited enzyme was assayed undiluted or diluted three, ten or 30 times (Fig. 2).

Inhibition of erythrocyte cholinesterase and reactivation of the inhibited enzyme. The titrimetric method was used for studying the kinetics of inhibition of erythrocyte cholinesterase by 1-naphthyl

N-methylcarbamate, 2-isopropoxyphenyl *N*-methylcarbamate and phenyl *N*-methylcarbamate. The activity of cholinesterase was measured at 3·0mM substrate concentration. Similar results were obtained with all the three inhibitors, the results obtained with 2-isopropoxyphenyl *N*-methylcarbamate being presented in Fig. 3.

When the concentration of inhibitor was small cholinesterase inhibition followed first-order kinetics up to approx. 1hr., whereas at high inhibitor concentrations deviations from first-order kinetics were observed already after 10min. inhibition. Rate constants for inhibition (k_i) were calculated from the first part of the inhibition curves according to the equation (1) for a bimolecular reaction with one component in excess (Aldridge, 1950; Aldridge & Davison, 1952), where c_i stands for the molar

$$k_i c_i t_i = \ln \frac{100}{a} \qquad (1)$$

concentration of inhibitor, t_i for the time of inhibition in minutes, and a for the activity of cholinesterase expressed as percentage of the control. The calculated k_i values are listed in Table 1.

After the inhibition deviated from first-order kinetics the reaction gradually approached a state of apparent equilibrium. Equilibrium constants (K) were calculated according to equation (2) from

$$K = \frac{a_e c_i}{100 - a_e} \qquad (2)$$

the activities of enzyme at equilibrium (a_e) expressed as percentage of the control. The mean of three or four enzyme activities determined at equilibrium was used for calculating the equilibrium constant at each concentration of inhibitor; K values given in Table 1 represent the means obtained at several inhibitor concentrations.

Since the equilibrium constant represents the ratio between the rate constant for spontaneous reactivation (k_r) and the rate constant for inhibition (k_i), the k_r value can be calculated from

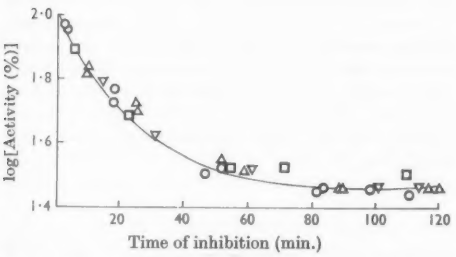

Fig. 2. Degree of inhibition of erythrocyte cholinesterase by 2-isopropoxyphenyl *N*-methylcarbamate measured by the spectrophotometric method after different dilutions of the inhibited enzyme preparation. Substrate concn.: 1·0mM; inhibitor concn.: 1·0μM. Enzyme dilution: ○, nil; △, threefold; □, tenfold; ▽, 30-fold.

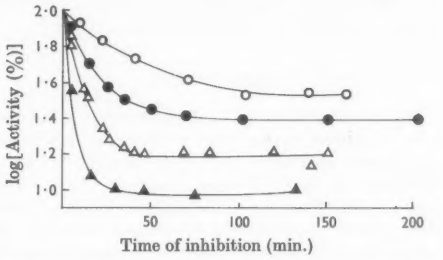

Fig. 3. Inhibition of erythrocyte cholinesterase by 2-isopropoxyphenyl *N*-methylcarbamate measured by the titrimetric method. Inhibitor concn. (μM): 0·5 (○), 1·0 (●), 2·0 (△), 5·0 (▲). Substrate concn.: 3·0mM.

Table 1. *Kinetic constants for inhibition of erythrocyte cholinesterase by monomethylcarbamates and for reactivation of the inhibited enzyme*

k_i (mol.$^{-1}$l. min.$^{-1}$) is the rate constant for inhibition calculated from the regression lines according to equation (1); constants are given ± the standard error of the slope of the line. K is the mean of the equilibrium constant calculated according to equation (2). k_r (min.$^{-1}$) calculated is the rate constant for spontaneous reactivation according to equation (3). k_r (min.$^{-1}$) obtained is the mean of the rate constants for spontaneous reactivation obtained experimentally; values are given ± the standard error of the mean. Numbers in parentheses indicate the number of individual estimates.

Inhibitor	$10^{-3}k_i$	10^6K	10^2k_r Calculated	10^2k_r Observed
1-Naphthyl *N*-methylcarbamate	17·9±0·95 (18)	0·794 (19)	1·42	0·90±0·48 (33)
2-Isopropoxyphenyl *N*-methylcarbamate	40±13 (17)	0·384 (18)	1·54	1·23±0·21 (6)
Phenyl *N*-methylcarbamate	0·2681±0·0069 (12)	54·3 (12)	1·46	1·30±0·98 (7)

equation (3), if K and k are known. For all the

$$K = \frac{k_r}{k_i} \qquad (3)$$

three inhibitors studied the calculated k_r values were practically the same (Table 1).

The rate of spontaneous reactivation was measured directly in the presence of 0·02M-substrate after the enzyme was treated with various concentrations of inhibitor. The rate constant for spontaneous reactivation (k_r) was calculated according to equation (4), where a_0 represents the activity

$$k_r = \frac{\ln(a_0 - a_1) - \ln(a_0 - a_2)}{t_2 - t_1} \qquad (4)$$

of the uninhibited sample, and a_1 and a_2 represent the activity of the inhibited sample at t_1 and t_2 min. after addition of substrate. As a_1 and a_2 were usually calculated for enzyme activities at 15 and 75min. after addition of substrate, the difference $t_2 - t_1$ corresponded to 60min. In Table 1, the mean k_r values obtained from several runs are given for each inhibitor studied. The experimentally obtained k_r constants were about 10–40% smaller than the calculated values.

DISCUSSION

Many authors studying cholinesterase inhibition have regarded carbamates as poor substrates of cholinesterases (Myers, 1952; Myers & Kemp, 1954; Wilson *et al.* 1960; Wilson, Harrison & Ginsburg, 1961; Brick, Brestkin & Yakovljev, 1964). Wilson *et al.* (1960) postulated a reaction scheme for the inhibition of cholinesterases by carbamates and the reactivation of the inhibited enzyme (equation 5),

$$E + I \xrightarrow{k_i} EI' \xrightarrow{k_r} E + \text{products} \qquad (5)$$

where E, I and EI′ stand for the free enzyme, inhibitor and carbamoylated enzyme respectively.

The results we have obtained for the kinetics of inhibition and spontaneous reactivation of erythrocyte cholinesterase by monomethylcarbamates can be represented by the same reaction scheme (equation 5), but the way in which k_i and k_r were obtained differed from that of Wilson *et al.* (1960). Whereas these authors obtained k_i and k_r from the derived differential equations comprising both the time-course of inhibition and the steady-state conditions, we calculated these constants separately: k_i from the first part of the inhibition curve and k_r from the equilibrium constant obtained from the inhibition curve at steady state (equations 2 and 3). This method of determination proved satisfactory, as the results obtained fit the mechanism proposed in equation (5).

According to equation (5) the inhibition of cholinesterase should follow first-order kinetics at the start of the reaction, as only a small amount of inhibited enzyme is present and the spontaneous reactivation is consequently small. This proved to be the case with all the three inhibitors studied. From the activity of enzyme as determined at steady state the equilibrium constant K can be evaluated, and from K and k_i the rate constant for spontaneous reactivation can be calculated (equation 3). As predicted from equation (5), k_r should be equal for all the three inhibitors used, since all yield monomethylcarbamoylated enzyme. The k_r values, both calculated and obtained experimentally, were in reasonably good agreement for all the three carbamates studied. However, values of k_r when obtained experimentally were somewhat lower than those calculated; this may be attributed to the presence of inhibitor during the measurement.

Winteringham & Disney (1964) studied the effect of substrate concentration, and the effect of dilution of the inhibited enzyme upon the measured degree of inhibition, and interpreted the interaction of cholinesterase with carbamates as a competitive reversible reaction. However, the authors assumed that under their experimental conditions their results could be interpreted from a hypothesis involving carbamoylation of the enzyme. In our studies 'initial' rate measurements have been made, as the time of contact of substrate and inhibited enzyme did not exceed 5min., and the dilution of enzyme was accomplished by adding an appropriately diluted substrate to the inhibited enzyme. Under these conditions the inhibition of enzyme by carbamates did not depend upon the concentration of substrate over a wide range (0·15M–0·1mM) and a dilution of inhibited enzyme up to 30 times did not affect the degree of inhibition. If the recovery of the enzyme activity is a result of dissociation of a reversible complex between the enzyme and the inhibitor, the k_r values should differ from one monomethylcarbamate to the other. Contrary to that, calculated and experimentally obtained k_r values in our studies suggest that the reversal of the enzyme–inhibitor complex plays only a minor part in the whole reactivation process, if any.

The authors are indebted to Dr M. Vandekar, Institute for Medical Research, Zagreb, and Dr W. N. Aldridge, Medical Research Council, Toxicology Research Unit, Carshalton, Surrey, for their stimulating interest and helpful advice during the experimental work, and valuable suggestions about the manuscript. This work was supported in part by research grants from the World Health Organisation, Geneva, and the Research Council of Croatia, Zagreb.

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The Determination of the Substitution Achieved at the α-Amino, ε-Amino and Imidazole Groups of Proteins with Special Reference to Derivatives of Gelatin

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(*Received 2 July* 1965)

1. The diminution of the quantity of pigment formed in the ninhydrin colorimetric reaction or the titre in two types of formol titration of a protein after substitution is used to determine the degree of substitution achieved at the various amino groups of a protein. 2. The determination of the substitution achieved at α-amino plus ε-amino groups by the ninhydrin colorimetric method as described by Cobbett, Gibbs & Leach (1964) has been modified to correct for the hydrolysis of the peptide chain which takes place during colour development. 3. The degree of substitution at the ε-amino groups was determined by the formol titration carried out at pH 9, essentially as described by the above authors. 4. Substitution at the α-amino plus ε-amino plus imidazole groups was determined from the diminution of the titre of a modified formol titration. This titration was carried out by adjusting the protein solution to pH 6·5 followed by the addition of formaldehyde and then titrating to pH 9·0. 5. The three methods have been applied to carbamoylated and benzenesulphonylated gelatin derivatives. The values for the degrees of substitution obtained by the ninhydrin (α-amino plus ε-amino) and the formol (pH 9·0, ε-amino) methods were shown to be almost identical. The values from the formol (pH 6·5–9·0, α-amino plus ε-amino plus imidazole) titration were consistently lower than the values obtained by the other methods and corresponded to no substitution having taken place at the imidazole groups by the preparative methods employed. 6. The application of the methods to other protein systems is discussed.

The study of the influence of substitution at the amino groups of a protein on its properties requires a knowledge of the level of substitution achieved in the sample being examined. Cobbett, Gibbs & Leach (1964) describe the application of three techniques for this purpose. The spectrophotometric method described by these authors depended on the increased u.v. absorption of the protein after substitution with a substituent containing a suitable chromophore. From this information together with a knowledge of the amino acid composition of the protein could be calculated the level of substitution achieved at the α-amino plus ε-amino, plus imidazole groups. The chromophoric requirement limits the application of the method and furthermore, as these authors point out, the method is subject to influence by other amino acids, particularly tyrosine, when they are present in significant amounts and are involved during the substitution reaction.

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These authors also describe the application of the formol titration, which under the conditions employed gives a measure of the substitution achieved at the ε-amino groups. The present paper describes a modification of this technique which enables the groups measured in the spectrophotometric method (α-amino plus ε-amino plus imidazole) to be examined.

The ninhydrin procedure, which is used for the determination of the substitution achieved at the α-amino plus ε-amino groups, was considered by Cobbett *et al.* (1964) to give low values, and these were attributed to the effects of hydrolysis which takes place during colour development. This method has now been modified to correct for these effects. Carbamoylated and benzenesulphonylated derivatives of gelatin are examined by this procedure together with the two types of formol titration and the results compared and discussed particularly with regard to the role of the imidazole groups in these derivatives.

METHODS

Carbamoylated gelatins. Stark, Stein & Moore (1960) have shown that cyanates react with the amino groups of proteins yielding carbamoyl derivatives. Whilst carbamoylation is not a simple substitution reaction, the product contains HN·CO– substituted for H– and it is convenient to refer to it as substitution. In the present work gelatin solutions were reacted with potassium cyanate under the conditions described in Table 1. The resultant solutions were gelled, cut into pieces (approx. $\frac{1}{4}$in. × $\frac{1}{4}$in. × 3in.) with a wire, and washed with flowing water in a beaker at room temperature for at least 24hr., the current of water being sufficient to keep the contents of the beaker stirred. The products were dried in a current of air and ground. The parent gelatins which were used for comparative purposes in the analyses were treated in an identical manner throughout except that the cyanate was omitted.

Benzenesulphonylated gelatins. Gelatin solutions were caused to react with benzenesulphonyl chloride at pH9 and 60°, the heating continued for 30min., the solutions gelled, and then treated as the carbamoyl derivatives. The only difference between samples was the amount of reagent used; up to twice that theoretically required to give complete substitution was employed. The parent gelatins were treated in an identical manner except that the reagent was omitted.

Moisture and ash. All the calculations were carried out on an ash and moisture-free basis. About 1g. of the material, accurately weighed, was melted with 10ml. of water and dried as a thin film for 18hr. at 105°. The loss in weight was recorded as the moisture content. A moisture dish with a lid was used to avoid gain of moisture in the desiccator. For the ash content about 1g. of material in a platinum dish was moistened with conc. H_2SO_4, charred over a Bunsen burner and heated at 650° in a muffle furnace for 2hr. and the residue weighed as ash. These techniques can readily be scaled down should the need arise.

Ninhydrin colorimetric method. This was carried out essentially as described by Cobbett *et al.* (1964), and is described here for convenience along with the present modification for the hydrolysis correction. The ninhydrin reagent was prepared as described by Moore & Stein (1948). The solution was prepared just before use or it had been stored for not more than 3 days in an O_2-free atmosphere in the dark.

Table 1. *Reaction conditions employed in the preparation of carbamoylated gelatins*

The weight of x% (w/w) solution of an alkali-processed gelatin used was 300g.

Sample no.	x(%, w/w)	KCNO (g.)	pH	Time (hr.)	Temp.
C1	10	4	7	1	50°
C2	10	4	8	1	50
C3	10	8	*	114	approx. 17
C4	20	16	7	5	50
C5	10	8	7	17	50
C6	20	16	*	114	approx. 17

* No pH adjustment; pH increases with time to about 9.

Recommended procedure. About 20–70mg. of the gelatin or gelatin derivative (about 20mg. for an unmodified gelatin to about 70mg. for a gelatin completely substituted at its α-amino plus ϵ-amino groups) was accurately weighed from a scoop into a 50ml. beaker. Water (25ml.) was pipetted into the beaker, the sample allowed to swell for about 30min. and then warmed and stirred at about 40° to bring it into solution. A portion (1ml.) of this solution was pipetted into a test tube; 2ml. of the ninhydrin reagent was added and the solutions were thoroughly mixed by swirling the tube. The tube was capped and placed in a bath of vigorously boiling water for 20min. The solution was cooled and mixed thoroughly and the extinction measured at 570mμ in a 0·5cm. cell against water. If a larger cell is to be used the solution is diluted with 5ml. of ethanol–water (1:1, v/v). Triplicate determinations were carried out for each sample and the reagent blank. The determinations on the parent and substituted gelatins were carried out simultaneously since the colour yield varied slightly with the age and batch of the reagent and to ensure that the development period was identical for both samples. An identical set of solutions prepared at the same time was heated for 40min. in the boiling water and the measurements were carried out as above (Recommended procedure; method 1). These values were used to calculate the hydrolysis correction. Experiments were also carried out in which an initial period of 25min., with correction periods of 25min. (method 2), or 25min. calculated from 15min. (method 3), was used. The results of all these experiments are in Table 2. During the development of the correction procedure, samples of a gelatin, a highly substituted gelatin and a reagent blank were heated under the above conditions for a range of heating periods; results of these experiments are in Fig. 1.

If the solution of the protein or its derivative was markedly different from pH5 so that it significantly changed the pH of the ninhydrin reagent, then either the solution was adjusted to pH5 before making up to volume or 4ml. of ninhydrin reagent was used. In the latter method, parent protein, derivative and blank solution were all treated in the same way. With gelatin or its derivative this procedure was required only when the material had both a low degree of substitution and a pH above 7 or below 4·5, i.e. its buffering capacity was large.

The degree of substitution, α, in the present work is defined as the proportion of the reacted amino groups to the total of the amino groups originally available, and is expressed as a percentage. The amino groups are those being measured by the particular analytical procedure employed, and thus the values obtained by different methods need not be the same. The significance of these differences is discussed later.

The calculation, which is as follows, is carried out in terms of extinction per unit weight of protein.

Parent gelatin:

Wt. of parent gelatin in 25ml.	$= M$ mg.
Protein (%) = 100 − (% ash + % moisture)	$= X$
Extinction of test solution after 20min.	$= Dp_{20}$
Extinction of test solution after 40min.	$= Dp_{40}$
Extinction of blank solution after 20min.	$= B_{20}$
Extinction of blank solution after 40min.	$= B_{40}$
Hydrolysis correction = $(Dp_{40} - B_{40}) - (Dp_{20} - B_{20})$	$= Hp$

Hence the colour yield per mg. of parent protein

$$= \frac{2500}{MX}(Dp_{20} - B_{20} - Hp) = Cp$$

Similarly for the substituted gelatin we have the colour yield per mg. of substituted protein

$$= \frac{2500}{NY}(Ds_{20} - B_{20} - Hs) = Cs$$

The degree of substitution, α, is given approximately by

$$\frac{(Cp - Cs)}{Cp} \times 100$$

This value is only approximately correct because no allowance has been made for the amount of substituent which has been added on to the protein, so that the protein in a given weight of sample is less than that used in the calculation.

The correct value is given by (Cobbett *et al.* 1964):

$$\alpha = \frac{(Cp - Cs)}{(Cp + kCs)} \times 100$$

where k is the maximum theoretical gain in weight for complete substitution of 1g. of dry, ash-free protein.

The evidence provided below indicates that the imidazole groups are not substituted by the benzenesulphonyl chloride or carbamoylated by cyanate under the conditions employed. The complete substitution of the α-amino and ϵ-amino groups (calculated from the work of Eastoe, 1955) in 1g. of gelatin (dry, ash-free), by benzenesulphonyl chloride would therefore increase the weight by 0·053g. It has been shown by Cobbett *et al.* (1964) that this reagent also reacts with the tyrosine, so that a value for k of 0·06 would be more accurate. For carbamoylation k is 0·02 (0·016g.).

Formol (pH 6·5–9·0) *titration.* A sample (0·5–3g.) of a gelatin or its derivative (about 0·5g. for an unmodified gelatin to about 3g. for a fully substituted gelatin) was accurately weighed into a 250ml. beaker containing 100ml. of water. The gelatin was allowed to swell in the water, brought into solution by warming at 40° and the beaker placed in a water bath maintained at 30±0·5°. The stirred solution was adjusted to pH 6·5 with a Radiometer automatic titrator. A portion (25ml.) of an approx. 10% (w/v) formaldehyde solution which had been previously adjusted to below pH 9 (pH 8·7–8·9) was added and the volume of approx. 0·02N-NaOH required to adjust the solution to pH 9·0 determined. A large volume of formaldehyde solution was prepared each day and stored in a beaker covered with a closely fitting plastic sheet containing a small hole for the pipette. The titration was carried out as rapidly as the response of the electrodes would allow to minimize side reactions. Blank determinations were carried out about every 2hr. with 100ml. of water that had been stored under the same conditions as the test solutions. This reading was subtracted from the other values before they were used in the calculations. For the most accurate work it is preferable to carry out the titration under N_2, any CO_2 having been swept from the solution. The results (Tables 3 and 4) indicate that the errors from this source cannot have been large. The calculation is carried out as described by Cobbett *et al.* (1964) for the formol titration carried out entirely at pH 9·0, i.e. Tp = ml. of 0·02N-NaOH/g. of protein (moisture and ash-free) of the parent gelatin. Ts = ml. of the corresponding titration for the gelatin derivative. The degree of substitution, α, is approximately

$$\frac{(Tp - Ts)}{Tp} \times 100$$

or correcting for the residues added to the protein as described for the ninhydrin method

$$\alpha = \frac{(Tp - Ts)}{(Tp + kTs)} \times 100$$

Formol (pH 9·0–9·0) *titration.* This was carried out essentially as described by Cobbett *et al.* (1964), and is determined as in the above method except that the initial pH of the solution before the addition of formaldehyde was 9·0. The calculation is carried out as above. The results are in Table 3.

RESULTS AND DISCUSSION

Ninhydrin colorimetric procedure. The method described by Cobbett *et al.* (1964) is based on the observations of Slobodian, Mechanic & Levy (1962), who showed that both the α-amino and ϵ-amino groups of a protein react quantitatively with ninhydrin. The quantity of pigment produced by the ϵ-amino groups is about 65–75% of that obtained

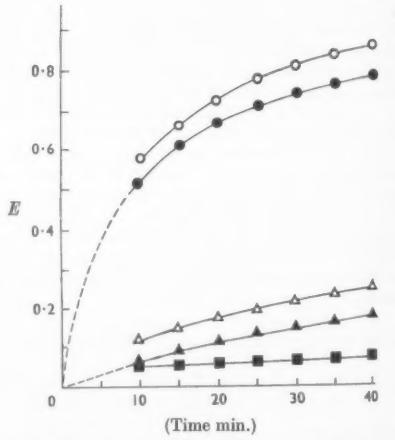

Fig. 1. Effect of the period of heating on the yield of pigment in the ninhydrin colorimetric procedure. Extinctions were measured in 0·5cm. cells against water at 570mμ. ○, 0·0201g. of parent gelatin in 25ml. of water. ●, 0·0201g. of parent gelatin in 25ml. of water, extinction corrected for reagent blank. △, 0·0646g. of carbamoylated gelatin in 25ml. of water. ▲, 0·0646g. of carbamoylated gelatin in 25ml. of water, extinction corrected for reagent blank. ■, Reagent blank. Calculated degree of substitution at α-amino plus ϵ-amino groups: 95·8% uncorrected; 98·5% corrected.

Table 2. *Degree of substitution at the α-amino plus ε-amino groups of gelatin as determined by the ninhydrin colorimetric procedure, with and without hydrolysis correction*

Values on the same horizontal line were determined in the same experiment. Method 1, 20 min./20 min.; method 2, 25 min./25 min.; method 3, 25 min./15 min.

Sample no.	Type of derivative	Type of gelatin	Initial heating period (min.)	Hydrolysis correction period (min.)	Degree of substitution (%)	
					Uncorrected	Corrected
C1	Carbamoylated	Alkali, hide	20	20	38·9	40·3
			25	25	38·0	39·1
			25	15*	39·5	39·3
C2	Carbamoylated	Alkali, hide	20	20	40·6	41·8
			25	25	38·4	38·3
			25	15*	41·1	40·6
C3	Carbamoylated	Alkali, hide	20		88·1	
			20		88·1	
			20	20	87·8	90·5
			25	25	87·6	90·2
			25	15*	87·4	89·5
C4	Carbamoylated	Alkali, hide	20		95·2	
			20	20	95·2	97·7
			25	25	94·1	97·7
			25	15*	93·4	96·0
C5	Carbamoylated	Alkali, hide	20		96·2	
			20		96·1	
			20	20	95·9	99·0
			20	20	95·8	98·8
			20	20	95·8	98·5
			25	25	95·7	98·8
			25	15*	94·8	97·8
C6	Carbamoylated	Alkali, hide	20		95·3	
			20	20	95·2	97·8
			25	25	94·4	97·7
			25	15*	94·0	96·8
BSC1	Benzenesulphonylated	Acid, pigskin	20	20	47·1	49·8
			25	25	44·0	45·6
BSC2	Benzenesulphonylated	Acid, pigskin	20	20	63·2	64·6
			20	20	64·3	65·7
			20	20	62·9	64·3
			25	25	63·3	66·5
BSC3	Benzenesulphonylated	Acid, pigskin	20	20	69·7	71·7
BSC4	Benzenesulphonylated	Acid, pigskin	20	20	75·1	77·8
BSC5	Benzenesulphonylated	Alkali, hide	20	20	93·6	95·1
BSC6	Benzenesulphonylated	Acid, pigskin	20	20	94·6	96·5

* Used to calculate 25 min. period correction.

from an equivalent quantity of an α-amino group. If the ε-amino group is on *N*-terminal or free lysine the figure is 7–10%. The imidazole groups do not react, nor do the amino groups when substituted. The diminution of the quantity of pigment formed after substitution is used to calculate the degree of substitution at the α-amino plus ε-amino groups. In most proteins, particularly those of high molecular weight, nearly all the colour arises from ε-amino groups and therefore the ninhydrin method should give very similar or identical values to those obtained by the formol titration carried out at pH 9. It was observed, however, by Cobbett *et al.* (1964) that the ninhydrin values tended to be lower and this was attributed to hydrolysis of the peptide chain during colour development. Although the method compares the protein with its derivative the hydrolysis effects are unlikely to cancel because the colour resulting from hydrolysis for a protein and a series of derivatives is not a fixed proportion of the total colour. The ratio of colour resulting from hydrolysis to that from

Table 3. *Degree of substitution at the ε-amino groups of gelatin determined by the formol* (pH 9·0–9·0) *titration, and at α-amino plus ε-amino plus imidazole groups determined by the formol* (pH 6·5–9·0) *titration*

Sample no.	Type of derivative	Type of gelatin	Degree of substitution (%) Formol (pH 9·0–9·0)	Formol (pH 6·5–9·0)
C1	Carbamoylated	Alkali, hide	39·2	33·1
			39·2	31·2
			38·6	
			38·9	
C2	Carbamoylated	Alkali, hide	40·6	33·6
			41·3	34·5
C3	Carbamoylated	Alkali, hide	90·2	77·4
			90·9	77·3
C4	Carbamoylated	Alkali, hide	98·9	86·6
			98·9	85·6
C5	Carbamoylated	Alkali, hide	99·8	90·3
			100·0	90·6
			100·0	
			99·0	
			98·7	
C6	Carbamoylated	Alkali, hide	98·7	84·0
			98·7	84·1
BSC1	Benzenesulphonylated	Acid, pigskin	49·0	43·4
			49·0	43·7
				45·5
BSC3	Benzenesulphonylated	Acid, pigskin	72·3	62·3
			71·6	60·3
				61·3
BSC4	Benzenesulphonylated	Acid, pigskin	78·6	67·8
			78·6	67·8
BSC5	Benzenesulphonylated	Alkali, hide	97·3	84·7
BSC6	Benzenesulphonylated	Acid, pigskin	98·8	84·2
			98·3	

Table 4. *Comparison of the values for the degree of substitution determined by the ninhydrin colorimetric method (corrected for hydrolysis), the formol* (pH 9·0–9·0) *titration and the formol* (pH 6·5–9·0) *titration*

The average values from Tables 2 and 3 are employed.

Sample no.	Method	Degree of substitution (%) Ninhydrin (*A*)	Formol (*B*) (pH 9–9)	Formol (pH 6·5–9)	Calc.* from (*A*)	Calc.* from (*B*)
	Groups determined ...	$(\alpha+\epsilon)$-NH_2	ϵ-NH_2	$(\alpha+\epsilon)$-NH_2 + imidazole	$(\alpha+\epsilon)$-NH_2 + imidazole	$(\alpha+\epsilon)$-NH_2 + imidazole
C1		40·3	39·0	32·2	35·3	34·2
C2		41·8	41·0	34·0	36·7	36·0
C3		90·5	90·5	77·4	79·3	79·3
C4		97·7	98·9	86·1	85·7	86·8
C5		98·8	99·5	90·4	86·7	87·3
C6		97·8	98·7	84·1	85·8	86·6
BSC1		49·8	49·0	44·2	43·7	43·0
BSC3		71·7	72·0	61·3	62·9	63·1
BSC4		77·8	78·6	67·8	68·2	68·9
BSC5		95·1	97·3	84·7	83·4	85·3
BSC6		96·5	98·5	84·2	84·6	86·4

* For calculation see the Discussion section.

α-amino plus ε-amino groups increases with the degree of substitution. In addition the hydrolysis rates of a protein and its derivative need not be the same.

Moore & Stein (1948) have shown that amino acids and some peptides yield all the pigment possible in the ninhydrin reaction within 20min. at 100°. Fig. 1 shows that with both gelatin and its derivative the quantity of blue pigment continues to increase after this period. The shape of the curve for an almost fully substituted gelatin derivative is almost a straight line, even over a total period of 40min., and indicates that most of the colour arises from α-amino groups released by hydrolysis of the peptide chain during colour development. The parent gelatin follows a similar course after 20–25min. when all the α-amino plus ε-amino groups already present in the material have completely reacted, leaving the α-amino groups resulting from hydrolysis as the sole source of increasing colour intensity.

In the present work the method of correcting for hydrolysis effects has been to heat a second set of colorimetric solutions for an equal additional period and to subtract the increase in extinction from that obtained during the first period. Such an approach had to be a compromise between the following: (*i*) the initial heating period must be sufficiently long to allow all the colour to develop from the α-amino plus ε-amino groups originally present in the sample; (*ii*) the additional heating period must be long enough to give a sufficient increase in extinction to allow accurate corrections to be applied; (*iii*) the total of (*i*) and (*ii*) must not be too great otherwise colour-fading effects become important. Three sets of initial/additional heating periods (methods 1, 2, 3, see the Methods section) were examined.

Method 1 gave values 1·2–3·1% higher (difference between actual values and not calculated as % of the original value) than the uncorrected values. The ratio of colour due to hydrolysis effects to the colour from groups already present increases with the degree of substitution and hence the errors increase from about 1·5% for half-substituted materials to about 3·0% for almost fully substituted derivatives. The values from method 2 (Table 2) were also close to the above two sets of values, whereas with method 3 the errors arising from the smaller increment in extinction were rather large. The presence of the opposing effects of colour fading and colour from hydrolysis effects is suggested by the similarities of the corrected and uncorrected values at low degrees of substitution with the 25min. initial period.

The reproducibility of method 1 was good, although it decreased slightly with the degree of substitution.

Formol titrations. Kenchington & Ward (1954 in their studies of the titration curves of gelatin alone at 40° showed that pH 6·5 is the most accurat boundary between the carboxyl group titration an the α-amino plus imidazole titration. At pH 6· less than 1% of the carboxyl groups remain to b titrated above this pH and are almost equal to an offset by the titration of the α-amino and imidazole groups remaining below this pH. Thus by adjusting the initial pH of the protein solution in the formol titration to pH 6·5 and then adding formaldehyde and measuring the quantity of alkali required to adjust the solution to pH 9, one obtains a measure of the α-amino plus ε-amino plus imidazole groups. The diminution of this titre on substitution is used to calculate the degree of substitution at these groups. The small amount of carboxyl group titration above pH 6·5 which remains after substitution results in a slight underestimate of the degree of substitution. Although Kenchington & Ward (1954) carried out their studies at 40°, the temperature of the formol titration was reduced to 30° to minimize side reactions. The results obtained show that no large errors resulted from this temperature difference (Table 4).

The formol titration which is carried out entirely at pH 9 does not measure all the ε-amino groups as some of them are already titrated at pH 9·0 in the initial pH adjustment (Kenchington & Ward, 1954; Kenchington, 1960). As a fixed proportion is titrated at this pH and the present method is a comparative one, the values for the degree of substitution will be unaffected by the procedure. By the use of pH 9·0 possible participation by imidazole groups is avoided.

The reproducibility of the formol (pH 9·0–pH 9·0) titration was similar to that of the ninhydrin procedure, and that of the formol (pH 6·5–pH 9·0) titration slightly poorer (Table 3). Values obtained by the former titration gave the expected close agreement with those from the modified ninhydrin method (Table 4).

Examination of derivatives. The values for the degree of substitution of the carbamoylated and benzenesulphonylated gelatins obtained by the formol (pH 6·5–pH 9·0) titration, which gives a measure of α-amino plus ε-amino plus imidazole, were considerably lower than those obtained by the other two methods and corresponded to no substitution having taken place at the imidazole groups. No reaction of cyanate with the imidazole groups has been reported in the papers by Stark *et al.* (1960) and Stark & Smyth (1963). Staab & Wendel (1960) have shown that the benzenesulphonyl derivative of imidazole is very rapidly hydrolysed at an alkaline pH. With the benzenesulphonylated gelatins the derivative which may have been formed with the imidazole in the early

stages of the reaction was unlikely to survive the complete preparation procedure, which employed a reaction period of 30min. at 60° and pH 9. It has also been shown by Gurin & Clarke (1934) that the imidazole groups were essentially unaltered during benzenesulphonylation, the derivative giving as intense a colour with the Pauly diazo reaction as the original gelatin.

From the work of Eastoe (1955, 1961) on the amino acid composition of gelatins and Courts (1954) on the *N*-terminal groups of gelatins, it can be calculated that imidazole represents 10·7–12·6% (on a residue basis) of the amino (α-amino plus ε-amino plus imidazole) groups of alkali-processed gelatins and 10·4–15·2% of those of acid-processed gelatins, with an average value for all the gelatins of 12·3%. By assuming the imidazole to be completely unsubstituted, the degree of substitution for the total amino groups has been calculated from the ninhydrin and formol (pH 9·0–pH 9·0) values by multiplying them by (100 − 12·3)/100. The results of these calculations, which are in Table 4, show very good agreement with the experimental values obtained by the formol (pH 6·5–9·0) titration. There is a tendency for the experimental values to be slightly lower than those of the calculated values, particularly when compared with the values from the alternative formol procedure. This can be attributed to the small amount of carboxyl titration which exists above pH 6·5 and which behaves in the procedure as unsubstituted amino groups.

Once it is established that the imidazole groups do not react in a particular preparative procedure, attention can be confined to the other amino groups and the ninhydrin technique employed, which requires only a few milligrams of the parent protein and its derivative. The additional advantage of the ninhydrin procedure is that a number of samples can be examined simultaneously. In cases where the imidazole groups do react the pH 6·5–pH 9·0 formol titration must be employed.

Application of methods to other proteins. The techniques described in the present work have been applied to gelatin derivatives and the following points would need to be considered before applying them to other proteins. If the number of amino groups per unit weight of protein is much greater than that for gelatin, then the quantity of protein used in the three techniques should be reduced, and vice versa. For the ninhydrin method curves of the type shown in Fig. 1 would indicate whether other colour-development times and hydrolysis corrections might be more appropriate than those used with gelatin and its derivatives. With non-gelling proteins the temperatures at which the formol titrations are carried out can be reduced, thereby further reducing the side reactions between the formaldehyde and the protein.

The lower limit of pH 6·5 used in one of the formol titrations needs consideration with other proteins. Kenchington & Ward (1954), in their study of the titration curves of gelatins, point out that this limit may not apply to other proteins unless the ratio of histidine to dicarboxylic acid residues is similar to that in gelatin. A study of the titration curve of the protein in the manner of these authors would probably give the required information. It would not be sufficient merely to move the boundary to a lower pH so that the carboxyl and imidazole titrations cancel, because, as mentioned above, when the protein is substituted the increased carboxyl titration above the boundary would behave in the procedure as unsubstituted amino groups.

The author is grateful to Mr J. A. Gibbs for some of the benzenesulphonylated gelatins and to Dr R. M. Laird for helpful discussions on the stability of benzenesulphonylated imidazole compounds. This paper is published by permission of the Director of Research and the Council of The Gelatine and Glue Research Association.

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Biochem. J. (1966) **98**, 513

Polyribosomes in Rat-Liver Preparations

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(*Received* 26 *July* 1965)

1. The distribution of rat-liver polyribosomes in sucrose density gradients has been investigated with regard to the effects of the preparative procedures and the physiological and pathological condition of the animal. 2. By using carefully defined conditions, three principal polyribosomal fractions have been isolated with $S_{20,w}$ values of 340, 275 and 225s in addition to the dimerized 120s and single 80s ribosomes. 3. The polyribosomes were very sensitive to treatment with ribonuclease and to mechanical stresses. 4. Incubation of dispersed hepatic cells and also cell-free preparations with puromycin in the presence of ATP and phosphoenolpyruvate caused rapid partial degradation of the polyribosomes. Treatment of the dispersed cells with actinomycin D also degraded the polyribosomes. 5. The liver polyribosomes of rats not raised under pathogen-free conditions and possibly of rats with an arthritic syndrome may be more fragile than those of healthy pathogen-free animals. 6. Treatment of pathogen-free rats with drugs stimulating liver anabolism profoundly affected the distribution of polyribosomes in sucrose density gradients.

The presence of polyribosomes, i.e. aggregates of single ribosomes held together by strands of RNA, has been demonstrated in many different cell types in various species. It is generally accepted that proteins are assembled on the constituent ribosomes of the polyribosomes although single ribosomes with attached messenger RNA may be capable of protein synthesis (Munro, Jackson & Korner, 1964; Lamfrom & Knopf, 1965). The polyribosomes are fragile structures that are extremely sensitive to minute quantities of ribonuclease and are easily broken by mechanical forces. It is uncertain therefore to what extent the polyribosomes isolated in the ultracentrifuge are fragments of larger structures degraded during cell disruption and the subsequent handling procedures. We have studied the effects of homogenizing techniques, the composition of the preparative media and the centrifugation procedures on large polyribosomal aggregates in rat-liver preparations. The influence of the condition of the animal, with regard to strain, sex, pathological state and the administration of certain drugs, on the polyribosomes has also been investigated. Finally, sucrose-density-gradient analyses have been carried out on extracts prepared from suspensions of dispersed rat-liver cells. Some of this work has been briefly described (Franklin & Godfrey, 1964).

MATERIALS AND METHODS

Animals. Two strains of rat were used, an albino strain and Norwegian hooded rats. Most of the albino and all of the Norwegian hooded rats were raised in a specific-pathogen-free animal colony (SPF rats). 'Non-SPF rats' refers to albino rats of the same strain as the SPF animals but raised in a colony not designed to exclude specific rat pathogens. All of the animals weighed 200–300g.

Media. A stock solution was prepared containing 0·25M-sucrose in 0·04M-tris–HCl buffer, pH 7·7. The following media were made by adding the required amounts (final concentrations quoted) of $MgCl_2$ and KCl: medium A, 4mM-$MgCl_2$ and 25mM-KCl; medium B, 10mM-$MgCl_2$ and 25mM-KCl; medium C, 1·5mM-$MgCl_2$ and 25mM-KCl; medium D, 0·1mM-$MgCl_2$ and 25mM-KCl; medium E, 4mM-$MgCl_2$ and 0·125M-KCl; medium F, 4mM-$MgCl_2$. Sucrose gradients were prepared linear over the range 10–25% (w/v) and were contained in 0·04M-tris–HCl buffer, pH 7·7; concentrations of KCl and $MgCl_2$ corresponding to those used in the homogenizing medium were also present.

Liver homogenates. The animals were killed by a blow on the head and bled freely. The liver was removed into the appropriate ice-cold medium. After being weighed, the tissue was minced with scissors and disrupted in 2·5 vol. of medium with a homogenizer. Three types of homogenizer were used: (*a*) a stainless-steel tube with a Perspex pestle having a clearance of 1mm. ('loose homogenizer'); (*b*) a Perspex tube and Teflon pestle with a clearance of 0·125mm. ('tight homogenizer'); (*c*) a stainless-steel tube with a

spherical Perspex pestle having a clearance of 0·063 mm. (Dounce homogenizer). Homogenizers (*a*) and (*b*) were driven by a low-torque air motor at 1000 rev./min.; (*c*) was operated by hand without rotation. After homogenization (see the Results section for details), the preparation was centrifuged either at 15000*g* for 15 min. or at 10000*g* for 10 min. to remove nuclei, mitochondria and debris. The required concentration of aqueous sodium deoxycholate was added to the supernatant (see the Results section), and the mixture was layered (0·1 ml.) over the sucrose density gradient in 5 ml. tubes. The gradient was centrifuged in the SW 39 rotor usually at 39000 rev./min. for 45 min. at 0–1°. Fractionation of the gradient was carried out by puncturing the tubes and collecting samples on a fraction collector. Usually 42 samples were collected and diluted fivefold before determining $E_{260m\mu}$.

Hepatic cells. The rat was killed, bled and the liver dropped into an ice-cold medium containing (per l.): NaCl, 9 g.; KCl, 0·42 g.; $NaHCO_3$, 0·2 g.; sodium citrate, 7·6 g. (medium 1). The organ was perfused with medium 1 by inserting the needle of a hypodermic syringe into the cut ends of the major hepatic vessels. The liver was left for 15 min. at 0°; this appeared to increase the yield of cells. After mincing with scissors, the tissue (4 g.) was dispersed in medium 1 minus sodium citrate (25 ml., medium 2) with 10 strokes by hand in the loose homogenizer; further 4 g. portions were disrupted as required and the suspension was filtered through a stainless-steel sieve (120 mesh). The sieved suspension was centrifuged for 3 min. at 100*g* at 0° and the sediment resuspended gently in medium 2 with a glass rod and left for 10 min. at 0°. The hepatic cells were finally centrifuged at 50*g* for 3 min. and resuspended in medium A (5 ml./8 g. of original tissue). The quality of the preparation was assessed by mixing 1 drop of the cell suspension with 1 drop of 0·5% (w/v) Trypan Blue in 0·9% (w/v) NaCl. Microscopic examination of a smear of this preparation showed well-dispersed intact hepatic cells and relatively few isolated nuclei. The liver cells were disrupted by homogenizing by hand in a glass tube with a smooth Teflon pestle (clearance 0·025 mm.) at 0°. After each stroke a sample was mixed with Trypan Blue in NaCl and examined microscopically. After 8 strokes about 90% of the cells were disrupted. The homogenate was centrifuged at 15000*g* for 15 min. at 0° and the protein content of the supernatant determined. Sufficient aqueous sodium deoxycholate was added to give 0·43 mg. of deoxycholate/mg. of supernatant protein. Density-gradient analysis was carried out at 39000 rev./min. for 45 min.

Measurement of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used, with crystalline bovine plasma albumin as standard.

Measurement of RNA. Ribosomal aggregates were precipitated with ice-cold 0·5N-$HClO_4$ and centrifuged. The precipitate was washed once and then extracted twice at 70° with 0·5N-$HClO_4$. The $E_{260m\mu}$ value was determined and the RNA concentration calculated assuming an extinction coefficient of 34·2 mg./cm.

Chemicals. Sodium deoxycholate was obtained from two sources: British Drug Houses Ltd., Poole, Dorset, and T. J. Sas and Sons, Ltd., Holborn, London. Puromycin hydrochloride was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Actinomycin D was kindly given by Merck, Sharp & Dohme Research Laboratories, Rahway, N.J., U.S.A. Atromid-S [ethyl 2-(*p*-chlorophenoxy)-2-methylpropionate] is a product of Imperial Chemical Industries Ltd. Cortisone acetate was obtained from Boots Pure Drug Co. Ltd., Nottingham. Crystalline pancreatic ribonuclease was obtained from British Drug Houses Ltd.

Induction of rat 'arthritis'. The right hind foot pads of SPF male albino rats were injected with 0·05 ml. of liquid paraffin containing a suspension of dead tubercle bacilli (5 mg./ml.) (Newbould, 1963). The injected foot swelled considerably during the next 4 days followed by a slight regression. Ten days after the injection there was an exacerbation of the swelling accompanied by inflammation and swelling of the other feet and other collagenous areas of the body (ears, tail etc.), which persisted for several weeks. The rats were severely debilitated during the course of the syndrome with extensive weight loss in mature rats and a failure to gain weight in growing animals (Newbould, 1963).

RESULTS

Studies on preparations from whole liver

Effect of homogenizing technique on polyribosomal distribution in density gradients. Livers of SPF albino male rats were disrupted in medium A with the loose homogenizer, by using 6 strokes at 1000 rev./min. After centrifuging at 15000*g* for 15 min. the supernatant was made 1·1% (w/v) with respect to sodium deoxycholate and subjected to density-gradient centrifuging at 39000 rev./min. for 45 min. This procedure is referred to below as the 'standard method'. The density-gradient pattern (Fig. 1*a*; 'control pattern') indicates five sharply defined components having approximate $S_{20,w}$ values of 80, 120, 225, 275 and 340s, calculated by the method of Martin & Ames (1961). Examination of the samples at 320 mμ revealed a peak at about 70s, presumably due to ferritin (Munro *et al.* 1964). The 'control pattern' was extremely reproducible when the procedure described above was closely followed. The homogenization procedure appears to be critical in determining the density-gradient profile. Thus 12 strokes with the loose homogenizer, 6 strokes with the tight homogenizer (both at 1000 rev./min.) or 6 strokes with the Dounce homogenizer resulted in the disappearance of the 225s, 275s and 340s components and a more polydisperse distribution of material sedimenting faster than the 120s ribosomes (Figs. 1*b*, 1*c* and 1*d*). Use of the tight homogenizer increased the height of the 80s (single) ribosomes. Resolution of the 120s and 80s ribosomes was lost after using the Dounce homogenizer. Three strokes with the loose homogenizer produced a pattern similar to the control but the material sedimenting between 120 and 225s was resolved into peaks at approx. 170 and 145s (Fig. 1*c*).

Effect of concentration of sodium deoxycholate on polyribosomal distribution. Identical control density-gradient patterns were obtained when sodium deoxycholate from two different suppliers

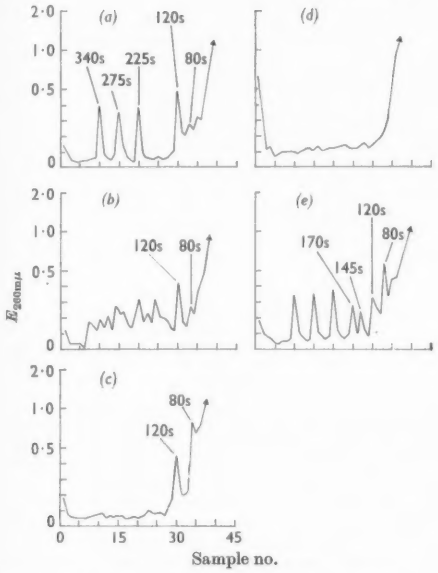

Fig. 1. Effect of homogenizing technique on sucrose-density-gradient profile of liver extracts from SPF albino male rats. (*a*) 6 strokes at 1000 rev./min. with 'loose homogenizer'; (*b*) 12 strokes at 1000 rev./min. with 'loose homogenizer'; (*c*) 6 strokes at 1000 rev./min. with 'tight homogenizer'; (*d*) 6 strokes by hand with Dounce homogenizer; (*e*) 3 strokes at 1000 rev./min. with 'loose homogenizer'. Centrifugation procedures were as described in the text.

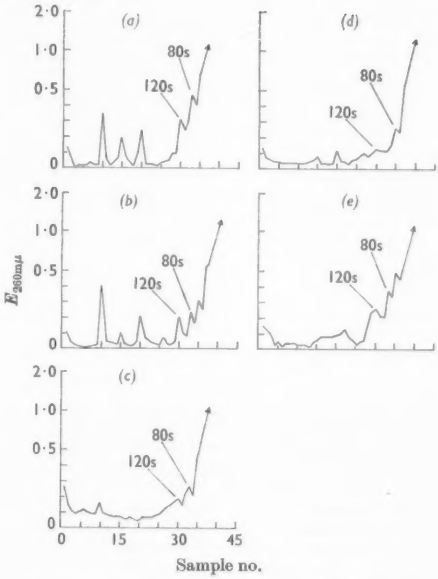

Fig. 2. Effect of concentrations of Mg^{2+} and K^+ on sucrose-density-gradient profiles of liver extracts from SPF albino male rats. (*a*) 1·5 mM-Mg^{2+} and 25 mM-K^+; (*b*) 10 mM-Mg^{2+} and 25 mM-K^+; (*c*) 0·1 mM-Mg^{2+} and 25 mM-K^+; (*d*) 4 mM-Mg^{2+} and no K^+; (*e*) 4 mM-Mg^{2+} and 0·125 M-K^+.

was used to treat the mitochondrial supernatants. The profiles were similar when the mitochondrial supernatant was made 0·5%, 1·1% or 1·3% with respect to deoxycholate but at a concentration of 1·66% all five peaks of the control pattern disappeared.

Effects of concentrations of Mg^{2+} *and* K^+ *on polyribosomal distribution.* Lowering the Mg^{2+} concentration to 1·5 mM (medium C) or raising it to 10 mM (medium B) had little effect on the gradient profile (Figs. 2*a* and 2*b*). At 0·1 mM-Mg^{2+} (medium D), however, the 120s, 225s, 275s and 340s peaks were considerably reduced in height (Fig. 2*c*). In the absence of K^+ (medium F) or at 0·125 M-K^+ (medium E) the polyribosomal peaks (225s, 275s and 340s) were also much smaller (Figs. 2*d* and 2*e*).

Effects of centrifugal force on polyribosomal distribution. (*a*) Preliminary centrifuging. Munro *et al.* (1964) removed nuclei, debris and mitochondria from liver homogenates before density-gradient centrifuging by centrifuging at 10000*g* for 10 min. Staehelin, Wettstein & Noll (1963) centrifuged for 10 min. at 20000*g*. We have found that the conditions of this preliminary centrifugation can affect the subsequent density-gradient profile. A liver homogenate prepared by the standard method from SPF male albino rats was centrifuged at 10000*g* for 10 min. The five components of the control pattern were present with an extra peak at 150s, but the definition of the peaks was reduced with the 'valleys' significantly higher than in the control pattern. Considerably more material sedimented to the bottom of the gradient (Fig. 3*a*). The overall amount of polyribosomal material appeared to be higher than when the homogenate was centrifuged at 15000*g* for 15 min. A similar effect was seen when the liver was disrupted with the Dounce homogenizer and centrifuged at 10000*g* for 10 min. (Fig. 3*b*).

(*b*) Prolonged centrifuging. Prolonged centrifuging may lead to a loss of resolution owing to the diffusion of the sedimenting components (Brakke, 1964). Thus when a 15000*g* supernatant, prepared by the standard method, was centrifuged through a gradient at 27000 rev./min. for 94 min. so that the product centrifugal force × time equalled that used

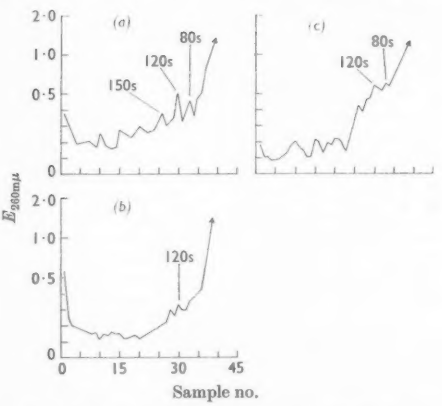

Fig. 3. Effect of centrifugation procedures on sucrose-density-gradient profiles of liver extracts from SPF albino male rats. (*a*) Liver homogenate prepared by 'standard method' and centrifuged at 10000*g* for 10min. before gradient analysis; (*b*) liver homogenate prepared with Dounce homogenizer and centrifuged at 10000*g* for 10min.; (*c*) liver homogenate prepared by standard method and subjected to density-gradient centrifuging at 27000rev./min. for 94min. at 0°.

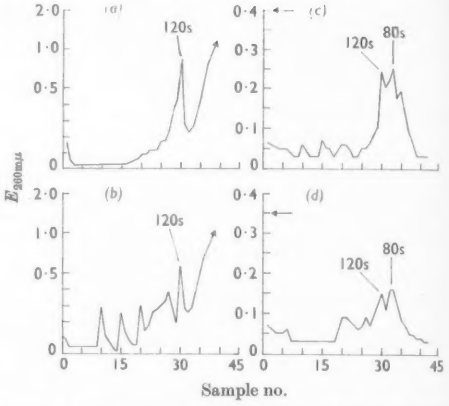

Fig. 4. Effect of treatment of liver extracts from SPF albino male rats with ribonuclease on sucrose-density-gradient profiles. (*a*) 15000*g* supernatant with 10μg. of ribonuclease/ml. at 37° for 5min.; (*b*) 15000*g* supernatant with 5μg. of ribonuclease/ml. at 0° for 30min.; (*c*) 340s fraction from gradient with 0·5μg. of ribonuclease/ml. at 0° for 30min.; (*d*) 225s fraction with 0·5μg. of ribonuclease/ml. for 30min. After enzyme treatment density-gradient analysis was performed at 39000rev./min. for 45min. The arrows in (*c*) and (*d*) indicate the $E_{260m\mu}$ of fractions 340s and 225s before treatment with ribonuclease.

to give the pattern shown in Fig. 1, the resolution of the five peaks was significantly reduced (Fig. 3*c*).

Properties of the polyribosomes and ribosomes of the control pattern

Protein/RNA ratios. The ribosomal and polyribosomal fractions were isolated from gradients and the protein and RNA contents determined. The protein/RNA ratios for the 80s, 120s, 225s, 275s and 340s fractions were found to be 1·00, 1·07, 1·05, 1·00 and 1·03 respectively.

Effect of ribonuclease treatment. When the 15000*g* supernatant was treated with pancreatic ribonuclease (10μg./ml.) for 5min. at 37° before the addition of sodium deoxycholate, the 225s, 275s and 340s fractions were extensively degraded and there was a marked increase in the height of the 120s peak (Fig. 4*a*). After isolation from the gradient the 340s and 225s fractions were degraded to 120s and 80s ribosomes by 0·5μg. of ribonuclease/ml. at 0° for 30min. (Figs. 4*c* and 4*d*). Treatment of the 15000*g* supernatant with 5·0μg. of ribonuclease/ml. at 0° (before deoxycholate treatment), however, had little effect on the density-gradient pattern subsequently obtained (Fig. 4*b*), except that there was more material between 170 and 140s.

Recentrifuging of polyribosomal fractions. The 340s, 275s and 225s fractions were isolated from gradients and diluted to reduce the sucrose concentration with medium A minus sucrose. Portions (0·2ml.) were layered over fresh gradients and centrifuged at 39000rev./min. for 45min. at 0–1°. Fractionation of the gradients showed that the 340s peak had split into a mixture of 340s, 275s, 225s, 150s, 120s and 80s fractions, the 275s peak into 275s, 225s, 150s, 120s and 80s fractions and the 225s peak into 225s, 150s, 120s and 80s fractions (Figs. 5*a*, 5*b* and 5*c*).

Effect of incubation of 15000*g supernatant with puromycin.* Williamson & Schweet (1965) found that incubation of reticulocyte polyribosome preparations with puromycin degraded the polyribosomes and increased the number of single and dimerized ribosomes. This effect was dependent on the addition of a source of 'high-energy' phosphate esters. The 15000*g* supernatant from the liver of SPF rats was incubated for 10min. with puromycin (0·4mM) in the presence and absence of ATP (1mM) and phosphoenolpyruvate (5mM) before the addition of deoxycholate. The mixture was then chilled and made 1·1% (w/v) with respect to sodium deoxycholate and centrifuged through a density gradient at 39000rev./min. for 45min. In the presence of ATP and phosphoenolpyruvate, puromycin partially degraded the 340s, 275s and 225s poly-

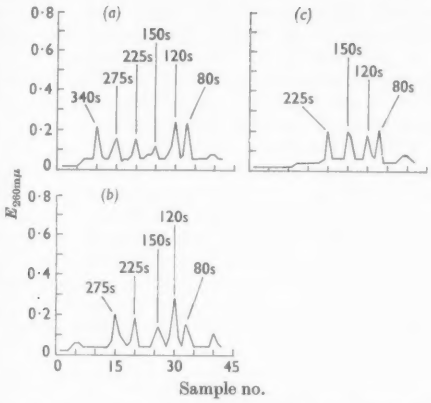

Fig. 5. Sucrose-density-gradient profiles obtained after recentrifuging of polyribosomal fractions isolated from gradient. (*a*) 340s fraction; (*b*) 275s fraction; (*c*) 225s fraction.

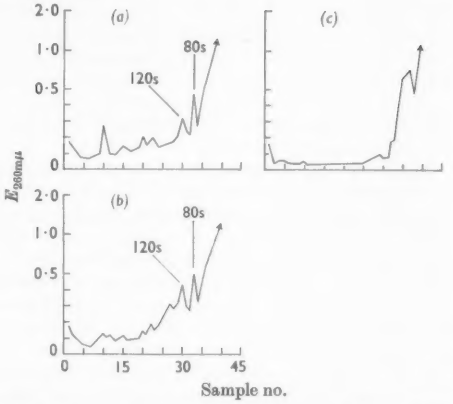

Fig. 6. Changes in sucrose-density-gradient profiles of liver extracts prepared by the 'standard method' from SPF albino male rats (*a*) 24hr., (*b*) 48hr. and (*c*) 10 days after injection of a suspension of dead tubercle bacilli in liquid paraffin into the right hind foot pad.

ribosomes, and markedly increased the 120–80s ribosomes and the material sedimenting at about 170–150s. Incubation with puromycin alone or with ATP and phosphoenolpyruvate in the absence of drug had little effect on the sedimentation profile.

Effect of sex and strain of rat on polyribosomes. Liver extracts were prepared by the standard method from female albino SPF rats. The density-gradient profile was very similar to that obtained from male rats. There was, however, a small peak sedimenting at about 370s that has not been observed in the liver preparations from male rats and considerably more material between 160 and 140s. Standard-method preparations were also obtained from the livers of SPF males of the Norwegian hooded strain. The polyribosomal distribution was very similar to that of the albino rats, although the 340s and 225s peaks were smaller and there was a marked peak at about 150s.

Liver sedimentation profiles from rats not raised under specific pathogen-free conditions. Liver extracts were made from non-SPF rats by the standard method. In the sedimentation profile the 80s and 120s ribosomes were evident but there was a broad distribution of material sedimenting faster than the single and dimerized ribosomes with no discrete polyribosomal peaks. However, when the livers from non-SPF rats were disrupted by 3 strokes with the loose homogenizer at 1000 rev./min., the 340s, 275s and 225s peaks appeared together with a component at about 150s.

Effect of development of arthritis syndrome. Within 24hr. of the injection of the suspension of dead tubercle bacilli in liquid paraffin into the foot pad there were striking changes in the sedimentation profiles of liver extracts prepared by the standard method. The polyribosomal components were less well defined and there was a small increase in the height of the 80s fraction (Fig. 6*a*). After 48hr. the polyribosomal peaks disappeared and there was ar increase in the 120s and 80s ribosomes (Fig. 6*b*). During the next 6 days there was a further decrease in the amount of polyribosomal material (Fig. 6*c*), and although the rats were kept for 32 days after the injection the sedimentation profiles did not return to the control pattern.

Effect of intraperitoneal inejction of cortisone acetate. There have been many reports describing the effects of cortisone on protein and RNA synthesis in the liver and there is general agreement that the hormone induces the new synthesis of hepatic enzymes (Feigelson & Greengard, 1962). It was therefore decided to investigate the effects of the administration of cortisone to the rat on the polyribosomal sedimentation profile of the liver. Cortisone acetate was given by intraperitoneal injection to SPF male albino rats in three doses of 5mg. each; the second injection was given 4hr. after the first and the third 16hr. after the second injection. The animals were killed 2hr., 24hr., 48hr. and 7 days after the last injection and liver extracts prepared by the standard method. The sedimentation profiles obtained after 2hr. showed striking changes from the control pattern; the 340s, 275s and 225s peaks disappeared and the height of the

80s fraction increased (Fig. 7*a*). After 24hr., however, the polyribosomal peaks began to reappear and this trend was maintained at 48hr. with a marked increase in the amount of material sedimenting between 180 and 150s (Figs. 7*b* and 7*c*). The control pattern had not returned 7 days after the injections (Fig. 7*d*).

Effect of administration of Atromid-S. The administration of Atromid-S to rats lowers the concentrations of total lipid and cholesterol in the blood and liver (Thorpe & Waring, 1962). An anabolic effect on the liver, which occurs within 3 days of daily administration of Atromid-S (0·25%, w/w, in powdered rat-food pellets) is due mainly to the deposition of protein (Dr D. S. Platt, unpublished work). Liver extracts were prepared by the standard method from SPF male albino rats after 5 days of administration of Atromid-S in the diet (0·25%, w/w). The sedimentation profile (Fig. 8) revealed a marked increase in the amount of material sedimenting between 180 and 130s and a greater proportion of material sedimented faster than 340s.

Sucrose-density-gradient profiles of extracts from dispersed hepatic cells. Suspensions of dispersed hepatic cells from SPF male albino rats were carefully homogenized in medium A as described in the Materials and Methods section. The 15000*g* supernatant was treated with sodium deoxycholate and subjected to density-gradient analysis. The pattern obtained was almost identical with the control pattern from whole liver (Fig. 9*a*).

Effect of incubation of hepatic cells with puromycin. Dispersed cells were incubated in medium A (8g. of original liver/5ml.) with 10μg. and 50μg. of puromycin/ml. for 30min. The drug caused extensive degradation of the polyribosomes and a considerable increase in the 120s and 80s ribosomes (Figs. 9*b* and 9*c*). Incubation of the cells alone had no effect on the density-gradient pattern.

Effect of incubation of hepatic cells with actinomycin D. Dispersed cells were incubated in medium A with 10μg. and 50μg. of actinomycin D/ml. Incubation was continued for 1hr. in 10μg. of actinomycin D/ml. and polyribosomes were progressively degraded with accompanying increase in the 120s and 80s ribosomes and material sedimenting between 170 and 140s (Figs. 10*a* and 10*b*). The higher concentration of drug completely degraded

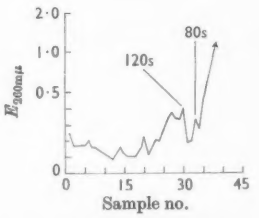

Fig. 8. Sucrose-density-gradient profile of liver extracts prepared by the 'standard method' from SPF albino male rats after 5 days of administration of Atromid-S in the diet (0·25%, w/w).

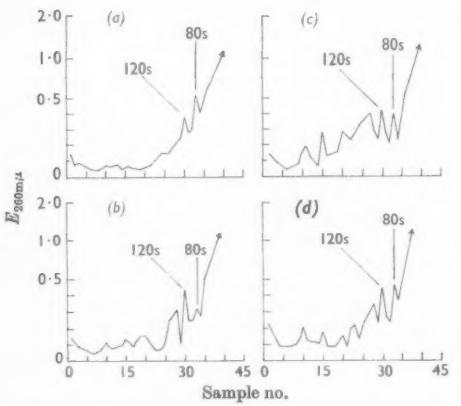

Fig. 7. Changes in sucrose-density-gradient profiles of liver extracts prepared by the 'standard method' from SPF albino male rats (*a*) 2hr., (*b*) 24hr., (*c*) 48hr. and (*d*) 7 days after intraperitoneal administration of cortisone acetate.

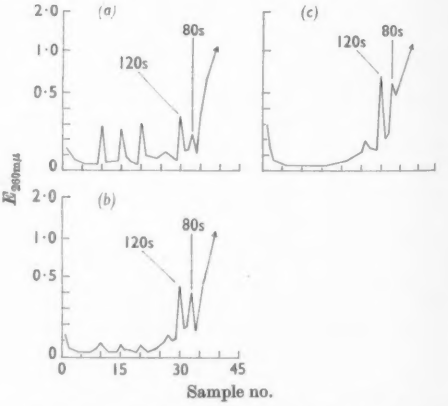

Fig. 9. Sucrose-density-gradient profiles of extracts from dispersed liver cells from SPF albino male rats. (*a*) Control preparation; (*b*) and (*c*) cells incubated with 10μg. and 50μg. of puromycin/ml. respectively for 30min. at 37° before disruption and density-gradient analysis.

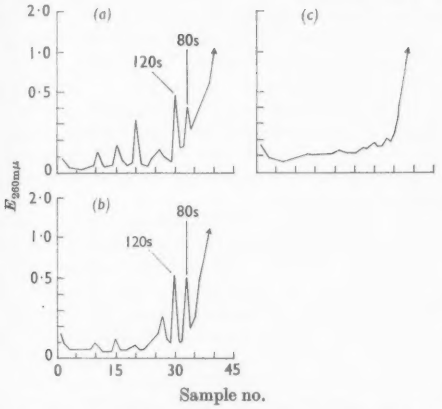

Fig. 10. Sucrose-density-gradient profiles of dispersed liver cells incubated at 37° with actinomycin: (*a*) 10 μg./ml. for 15 min.; (*b*) 10 μg./ml. for 1 hr.; (*c*) 50 μg./ml. for 15 min.

the polyribosomal and ribosomal components within 15 min. (Fig. 10*c*).

DISCUSSION

There is no evidence as to whether the discrete polyribosomal aggregates described in this paper with $S_{20,w}$ values of 225, 275 and 340s (and possibly 150s, which may be an aggregate of 120s and 80s ribosomes mediated by Mg^{2+}; Peterman, 1960) exist in liver cells of the living rat. Because of their great sensitivity to ribonuclease and mechanical stress the study of polyribosomes by homogenization and centrifuging is fraught with possible artifacts. The sedimentation properties of large macromolecules in centrifugal fields impose other difficulties. The experiments described here clearly demonstrate the way in which the $E_{260m\mu}$ sedimentation profiles of liver-mitochondrial supernatants depend on the technical procedures used and on the physiological or pathological condition of the animal.

The 'control' pattern we obtained for the distribution of polyribosomes in sucrose gradients differs considerably from patterns reported by other workers for liver preparations (Staehelin *et al.* 1963; Munro *et al.* 1964). The sharply defined components at 225, 275 and 340s have not been described elsewhere. The appearance of these components was dependent on the severity of the procedure used to disrupt the whole liver, suggesting considerable fragility in their structure. It is unlikely that these discrete components are stabilized by fragments of endoplasmic reticulum since they were stable over a range of concentrations of deoxycholate (0·5–1·33%). A higher concentration of deoxycholate (1·66%) degraded the 120s and 80s ribosomes as well as the polyribosomes.

The polyribosomes were stable in a wide range of Mg^{2+} concentrations, suggesting they are not aggregates caused by non-specific magnesium binding. The Mg^{2+} ion appears to be important in stabilizing the polyribosomal structure since the peaks disappeared in 0·1 mM-Mg^{2+}. Munro *et al.* (1964) have also reported that rat-liver polyribosomes are unstable in 0·1 mM-Mg^{2+}. Though the concentration of K^+ was also important in maintaining the integrity of the polyribosomes the effects of this ion were not studied in detail, so that its role remains unclear.

Strong evidence that the 225s, 275s and 340s fractions are polyribosomal aggregates linked together by a labile RNA molecule was provided by the effects of ribonuclease. The polyribosomes were much more sensitive to ribonuclease after isolation from the sucrose gradient. A high concentration of ribonuclease (10 μg./ml.) at 37° was required to degrade the polyribosomes before treatment of the mitochondrial supernatant with deoxycholate. It is likely therefore that the endoplasmic reticulum in some way protects the polyribosomal structure against ribonuclease attack. Alternatively, the resistance of polyribosomes in the mitochondrial supernatant to ribonuclease could be due to the presence of some inhibitor of the enzyme such as that described by Shortman (1961).

A significant proportion of polyribosomal material may be sedimented during the preliminary centrifuging of the homogenate. Centrifuging at 10000*g* for 10 min. reduced the resolution of the 225s, 275s and 340s peaks as well as increasing the overall amount of polyribosomes in the gradient. In this context Brakke & Daly (1965) have discussed the loss in resolution of components in density-gradient centrifuging when the concentration of the sedimenting components is increased. A further fall in the resolution of the polyribosomal peaks was evident in our experiments when the period of density-gradient centrifuging was extended and the centrifugal speed reduced. A similar effect has been reported by Brakke (1964) in a study of the effect of centrifugation conditions on the sedimentation of virus particles in sucrose-density gradients. To obtain high resolution it is important to use a low macromolecular concentration and maximal centrifugal force with the minimum period necessary for an adequate movement of the components along the gradient.

The partial degradation of the polyribosomal fractions into smaller fragments that occurs during isolation and recentrifuging suggests that rupture

may occur at specific weak points in the polyribosomal structure. Rifkind, Luzzato & Marks (1964) have described large polyribosomes containing up to 20 single ribosomes in intact reticulocytes. These structures were characterized by exposed lengths of messenger RNA appearing between clusters of ribosomes. They suggested that the preponderance of much smaller polyribosomes in sucrose-density gradients of reticulocyte lysates may be due to the rupture of larger aggregates at the sites of exposed messenger RNA. It is possible that similar intervals between ribosomal clusters may occur along the length of the liver polyribosomes, which could account for the peculiar manner in which these structures fragment during isolation from the gradient and recentrifugation.

The interpretation of the partial degradation of the polyribosomes to 120s and 80s ribosomes during incubation of the mitochondrial supernatant with puromycin in the presence of ATP and phosphoenolpyruvate is uncertain. Williamson & Schweet (1965) found that incubation of reticulocyte polyribosomes with puromycin caused a similar degradation. In contrast with their findings with reticulocyte preparations, however, we observed only very minor degradation of the liver polyribosomes in the absence of puromycin. Williamson & Schweet (1965) found an extensive conversion of reticulocyte polyribosomes into 80s ribosomes under these conditions, which was only slightly accelerated by puromycin. The pronounced acceleration by puromycin of the degradation process in the rat-liver preparations might be attributed to the stripping away of the nascent polypeptides by the antibiotic. It is possible that the nascent polypeptides in some way stabilize liver polyribosomes, although Williamson & Schweet (1965) have discounted this possibility in reticulocyte polyribosomes.

Not surprisingly, there were minor differences in the sedimentation profiles between liver preparations from rats of different sexes and strains. The failure to obtain the discrete polyribosomes by the standard method from non-SPF animals is puzzling. Since they were demonstrable when the severity of homogenization was reduced, the liver polyribosomes of the non-SPF rats may be more fragile than those of SPF rats. Alternatively, some property of the liver of SPF rats might protect the polyribosomes during disrupting of the cells. Similar considerations could apply to the changes in the sedimentation profile that occur during the development of the rat arthritis syndrome, during which the animals are severely debilitated. It is not clear whether there is a fall in the polyribosomal content of the liver cells or whether there is an increased sensitivity to homogenization.

Under conditions of increased protein synthesis in the liver, induced by the administration of cortisone or Atromid-S, there were marked changes in the sedimentation profiles. There was no obvious reduction in the total amount of polyribosomes, but rather a redistribution of material in the gradient. Again the significance of these results is not clear, but in some way they may reflect the changes in protein and RNA synthesis in the liver induced by drug administration.

The experiments with dispersed hepatic cells underline the fragility of the discrete polyribosomal aggregates. Before reproducible sedimentation profiles could be obtained it was necessary to control carefully the procedure for the disruption of the cells. More vigorous homogenization or ultrasonic disruption of the cells degraded the polyribosomes and greatly increased the height of the 120s and 80s peaks. The rapid degradation of the polyribosomes and 120s and 80s ribosomes produced by incubation of the cells with a high concentration of actinomycin D (50μg./ml.) suggests, in accordance with Revel & Hiatt (1964), that the drug may induce polyribosomal fragility with consequent disintegration during homogenization. This effect is thought to be in addition to the inhibition of RNA synthesis by actinomycin D. The degradation of the polyribosomes induced by incubation of the cells with puromycin resembles the effect of this drug observed by Williamson & Schweet (1964) on intact reticulocytes.

It is not yet clear whether the polyribosomes described in this paper, or indeed in other published reports of polyribosomes in disrupted cell preparations, are a true indication of the size and nature of polyribosomes in the intact cell. Preliminary experiments suggest that there is not necessarily any obvious correlation between the type of sedimentation profile and the rate of protein synthesis *in vivo* or *in vitro*. The functional significance of the polyribosomes described here is therefore uncertain. However, our results emphasize the difficulties in the interpretation of polyribosomal sedimentation patterns.

We are grateful to Dr B. B. Newbould and Mr R. Eamonson for the injection of rats with tubercle bacilli in liquid paraffin.

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The Hypobranchial Mucin of the Whelk *Buccinum undatum* L.

THE POLYSACCHARIDE SULPHATE COMPONENT

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(*Received* 2 *September* 1965)

1. A polysaccharide sulphate has been isolated from the hypobranchial mucin of the whelk *Buccinum undatum*. 2. The molecular weight of this polysaccharide, which is a glucan carrying one ester sulphate group per monosaccharide residue, is $1{\cdot}7 \times 10^5$. 3. Some investigations bearing on the location of the ester sulphate groups are reported. 4. The viscosity of the whole mucin has been shown to depend mainly on the glucan sulphate.

The hypobranchial mucin of the whelk *Buccinum undatum* L. consists of a glycoprotein in loose association with a polysaccharide sulphate (Hunt & Jevons, 1963, 1965*a*). The polysaccharide, which has been shown previously to contain glucose as its only monosaccharide constituent, has now been isolated in a pure form and its structure investigated.

Polysaccharide sulphates composed of ester-sulphated neutral monosaccharide units are unusual in higher animals, in which sulphate-carrying polysaccharides commonly contain hexosamine and hexuronic acid (e.g. the chondroitin sulphates or heparin). Sulphated neutral polysaccharides occur commonly among the marine algae (Dillon, 1954; Ross, 1953) as structural polysaccharides and are sporadically distributed among the marine fauna either as epithelial secretions or as structural polysaccharides occupying the place of the chondroitin sulphates, hyaluronic acid or keratosulphate (Lash & Whitehouse, 1960; Bettelheim-Jevons, 1958; Stacey & Barker, 1962).

MATERIALS AND METHODS

Preparation of the polysaccharide sulphate. A solution (1%, w/v) of salt-free mucin, in water, was brought to 60° and an equal volume of 90% (w/v) phenol in water was added slowly with stirring. The mixture was stirred at 60° for 30min. and then cooled to 4° and allowed to stand at this temperature overnight in the cold-room. During this period the mixture separated into three layers: a lower phenol layer, an upper aqueous layer (containing the polysaccharide sulphate) and a layer of denatured protein at the interface. Most of the lower phenol layer was withdrawn, by using a large hypodermic syringe with a long needle. The remaining aqueous phase and denatured protein were centrifuged at 20000*g* at 4° for 30min. The residual phenol phase was again withdrawn and the aqueous phase then decanted from the protein precipitate. The aqueous phase was freeze-dried for 16hr. to ensure complete removal of phenol and to yield a pure white product.

It was found that the separated polysaccharide sulphate material could be selectively precipitated by the addition of KCl. Precipitation as a final purification step was carried out from aqueous 1% (w/v) solutions of the polysaccharide sulphate by addition of solid KCl (to a final concentration 0·2M) and then allowing the solution to stand at 4° for 6hr. The precipitate was collected by centrifugation at 20000*g*, redissolved by addition of water at room temperature, dialysed against repeated changes of distilled water for 36hr. and freeze-dried. It was considered a measure of the homogeneity of the material that the precipitation took place completely within a fairly narrow concentration range (i.e. 0·2±0·03M-KCl). The supernatant solution after removal of the precipitate did not contain organic material.

Physical techniques. Analytical ultracentrifugation was carried out with a Spinco model E ultracentrifuge in the Department of Biochemistry at the Manchester College of Science and Technology, by kind permission of Professor A. A. Eddy and with the aid of Mr G. McBride.

Infrared spectra were measured with either a Unicam SP.200 recording spectrophotometer or a Perkin–Elmer model 28 recording spectrophotometer. Samples were examined, either as mulls in Nujol (liquid paraffin) or as freeze-dried films, between polished NaCl plates.

Molecular weights were determined either by the Archibald approach to sedimentation equilibrium method (Archibald, 1947; Klainer & Kegeles, 1955) or by a combination of sedimentation and diffusion coefficients (Schachman, 1957). Sedimentation coefficients at infinite dilution were determined by extrapolation to zero concentration after determination of the sedimentation coefficients at 0·1, 0·25, 0·5 and 1·0% (w/v) concentration in 0·02M-phosphate buffer, pH 7·9 (I 0·15 with respect to NaCl), and 59780rev./min. (260000*g*). Diffusion coefficient determinations were carried out at 0·5% (w/v) concentration in the same buffer, with a Perkin–Elmer model 38A apparatus fitted with a schlieren optical system. Results were evaluated by the 'height–area' method of Lamm (1937).

Viscosity measurements were carried out in Ostwald viscometers of 3ml. capacity.

Analytical techniques. The techniques of qualitative and quantitative carbohydrate analysis were exactly as de-

scribed in Hunt & Jevons (1965*a*). D-Glucose was identified with D-glucose oxidase (Glucostat, Worthington Biochemical Corp., Freehold, N.J., U.S.A.).

Estimation of ester sulphate was carried out according to the procedure of Dodgson & Price (1962), the same technique being applied to the measurement of the rate of release of sulphate on dilute acid hydrolysis.

Materials. Monosaccharides, cellobiose and chondroitin sulphate were obtained from British Drug Houses Ltd., Poole, Dorset. Laminarin was obtained from H. Gurr Ltd., London. λ- and κ-Carrageenins were gifts from Dr C. H. Wynn of the Department of Chemistry, University of Manchester.

RESULTS

Physical characterization. The sedimentation pattern produced by centrifugation of the polysaccharide sulphate at 59780rev./min. (260000*g*) at 1% (w/v) concentration in phosphate buffer, pH7·9 (*I* 0·15 with respect to sodium chloride), at 20° is shown in Fig. 1. Only one sedimenting component is apparent, the peak being symmetrical albeit hypersharp. The self-sharpening property of the material on sedimentation is apparently a function of the high viscosity. The material had $S^{1\%}_{20}$ $5{\cdot}25\times10^{-13}$ and S^{0}_{20} $9{\cdot}75\times10^{-13}$, partial specific volume $\bar{v}_{20}$ 0·6 and the diffusion coefficient $D^{0{\cdot}5\%}_{20}$ $3{\cdot}28\times10^{-7}$. The mol.wt. calculated from S^{0}_{20} and $D^{0{\cdot}5\%}_{20}$ was $1{\cdot}72\times10^{5}$ and from the approach to sedimentation equilibrium method $1{\cdot}70\times10^{5}$ and $1{\cdot}10\times10^{5}$ at the meniscus and base respectively. The reduced viscosity at aqueous 0·25% (w/v) concn., at 25°, was 21320ml./g. and the intrinsic viscosity at the same temperature in 0·5M-sodium chloride was 500ml./g.

The infrared spectrum of the material is shown in Fig. 2. Apart from the absorption frequencies due to carbohydrate and sulphate, discussed below, the following bands were noted. The intense absorption at 3390cm.$^{-1}$ represents the hydrogen-stretching frequency arising from the polysaccharide hydroxyl groups. Normally this frequency would be in the range 3700–3510cm.$^{-1}$; its presence at 3390cm.$^{-1}$ implies a considerable degree of hydrogen bonding involving the polysaccharide hydroxyl groups.

The absorption frequency at 1645cm.$^{-1}$ arises not from carboxyl groups, which are almost totally absent, but from absorbed water which here is an integral part of the crystal of the polysaccharide molecules. This frequency has been shown by Forgiath & Rowen (1951) to be present in the spectrum of bacterial cellulose and was shown here to be present in the spectra of starch, cellulose, yeast mannan and laminarin.

Chemical characterization. Paper chromatography of acid hydrolysates of the material indicated that the only monosaccharide present was glucose and qualitative examination with glucose oxidase (Glucostat), according to the procedure described in the Nutritional Biochemicals Corp. method sheet for this reagent, showed this to be D-glucose. The polysaccharide can therefore be designated as a glucan.

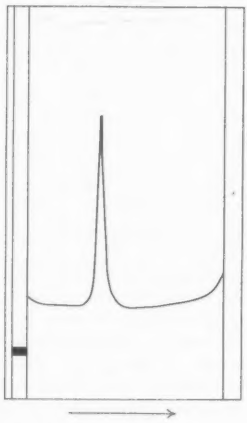

Fig. 1. Sedimentation pattern of the glucan sulphate after 128min. at 59780rev./min. (260000*g*) in 0·02M-sodium phosphate, 0·15M-sodium chloride buffer, pH8·0. Concentration of glucan sulphate, 1% (w/v); temperature, 20°.

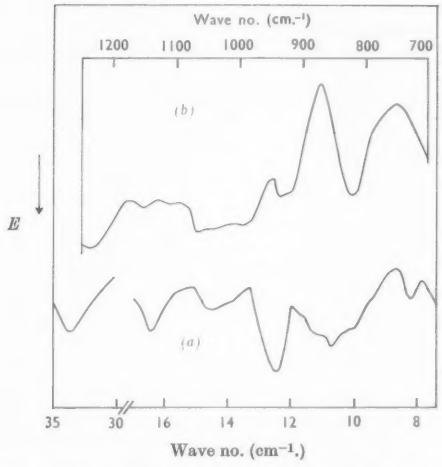

Fig. 2. Infrared spectra of the glucan sulphate: (*a*) as a freeze-dried film; (*b*) (inset) as a Nujol mull. Scale expansion ×2 of the region 700–1250cm.$^{-1}$.

Analysis of the materials for hexose as glucose (modified orcinol reaction; Hunt & Jevons, 1965*a*) gave 54% (w/w), and for sulphate as SO_4^{2-} 30% (w/w). The moisture content of the freeze-dried material was 13% (w/w) and the peptide content, determined by quantitative amino acid analysis, was 2% (w/w).

Structure of the polysaccharide. (i) Desulphation; the infrared spectrum. In order that the specific infrared absorptions of the carbohydrate structure might be investigated without interference from the ester sulphate frequencies, desulphation of the glucan sulphate was carried out according to Kantor & Schubert (1956), by using methanol and acetyl chloride. Yields were not estimated because of difficulty experienced in recovering material from the reaction vessels completely. After two desulphation treatments no sulphate could be detected in the polysaccharide material. Fig. 3 shows the infrared spectrum of the material after desulphation (cf. the sulphated material, Fig. 2).

(ii) Colour reactions. Neither the polysaccharide sulphate nor the desulphated polysaccharide gave any colour reaction with a solution of iodine in potassium iodide (10%, w/v, iodine in aq. 20%, w/v, potassium iodide), thus rendering it unlikely that the linkages between the glucose residues are of the α-(1→4)- type. When the desulphated glucan was treated with chlor-zinc-iodine reagent (obtained from British Drug Houses Ltd., Poole, Dorset, under the name Schultze's solution; Egami *et al.* 1955) the particles of material, which is insoluble in water, showed a deep-blue colour (transmitted-light viewing) exactly comparable with that produced by the same reagent with particles of Whatman cellulose powder.

(iii) Degradation by cellulase. The micro-organism *Myrothecium verrucaria* (strain 45541) produces an enzyme with a specificity for the hydrolysis of the β-(1→4)-linkages of cellulose. This enzyme may be obtained in culture filtrates of the organism (Selby, Maitland & Thompson, 1963). A culture filtrate was kindly provided by Dr K. Selby of the Shirley Institute, Didsbury, Manchester. The solution was free of low-molecular-weight material, 0·1 M with respect to sodium chloride, 0·01 M with respect to sodium phosphate at pH 6·4 and saturated with chloroform as preservative. The procedure, according to K. Selby (personal communication), for incubation with the enzyme was as follows. To 1·5 mg. of the desulphated glucan was added 5·0 ml. of the enzyme solution and the mixture incubated at 30° for 24 hr. Samples of cellulose [β-(1→4)-], amylose [α-(1→4)-] and laminarin [β-(1→3)-] were also incubated with the enzyme. After incubation the enzyme action was halted, by heating to 90° for 10 min., and the solutions were centrifuged at 9000*g* for 5 min. Measurement of the increase of reducing power according to Somogyi (1952) indicated that degradation by the enzyme had occurred in both the glucan and cellulose but not in either the amylose or the laminarin. Of the available reducing groups calculated to be present in the glucan, 66% had been released. The glucose content of the desulphated glucan is 96% (w/w). The supernatant from the glucan and the cellulose digests were desalted, concentrated and examined by paper chromatography (Hunt & Jevons, 1965*a*) with glucose and cellobiose as standards. Staining with aniline hydrogen phthalate showed that degradation of both the glucan and the cellulose to glucose and cellobiose had occurred.

(iv) Partial acid hydrolysis. The glucan was subjected to partial acid hydrolysis. Glucan (1·0 mg.) was dissolved in 10 ml. of 0·1 N-hydrochloric acid by heating under reflux at 100°. Refluxing was continued for 4 hr. and, after removal of the acid *in vacuo*, the hydrolysate was redissolved in water (0·1 ml.) and examined by paper chromatography. Partial acid hydrolysis yielded sugars with the R_F values of glucose, cellobiose and a smaller proportion of higher oligosaccharides.

Position of the sulphate residues. Barium sulphate was not precipitated by the addition of barium chloride solution to solutions of the glucan sulphate. Mild acid hydrolysis (1 N-hydrochloric acid for 1 hr. at 100°), however, of the glucan sulphate released sulphate precipitable as barium sulphate. Thus the sulphate residues were apparently covalently bound to the polysaccharide.

(i) Infrared spectra. The infrared spectra of the glucan sulphate (Fig. 2) show the presence of intense absorptions at 1240 cm.$^{-1}$ and over the

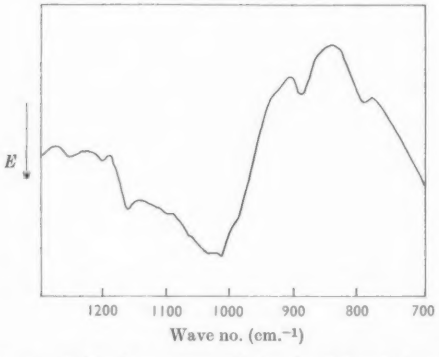

Fig. 3. Infrared spectrum of the desulphated glucan sulphate, as a Nujol mull, in the range 700–1300 cm.$^{-1}$.

range 800–840cm.$^{-1}$. On desulphation these two bands disappear entirely (Fig. 3) indicating ester sulphate (Hoffman, Linker & Meyer, 1958). Apart from these spectral changes it is to be noted that desulphation also brings about the disappearance of absorptions at 940cm.$^{-1}$, 920cm.$^{-1}$ and 1000cm.$^{-1}$. Examination of the infrared spectra of λ-carrageenin, κ-carrageenin and chondroitin sulphate A before and after desulphation showed similar changes. The possibility that spectral changes were arising in part from structural changes in the polysaccharide itself, brought about by the desulphation reaction and resulting in the formation or destruction of 3,6-anhydroglucose units, was obviated by testing for 3,6-anhydro-sugars (O'Neill, 1955) in both the sulphated and desulphated glucans. The tests on both materials were negative.

(ii) Rate of hydrolysis of the sulphate groups. With the procedure of Rees (1963) it was found that release of ester sulphate residues from the glucan sulphate, in 0·25N-hydrochloric acid at 100°, proceeded linearly according to a plot of $\log(a/a-x)$ versus t (where a is the total sulphate present and x the amount released after time t). The half-time of this reaction was calculated to be of the order 0·41hr.

(iii) Potassium chloride precipitation. As already stated the glucan sulphate was readily precipitable below 4° by addition of potassium chloride to a concentration of 0·2M. The solubility properties of the freshly formed precipitate and of the centrifuged consolidated precipitate differ, the former redissolving in the 0·2M-potassium chloride immediately on allowing it to come to room temperature whereas the latter remained completely insoluble in the 0·2M-potassium chloride at room temperature and could moreover be redissolved in distilled water, at room temperature, only with difficulty. The property of precipitation by potassium chloride may be related to the position and configuration of the sulphate groups on the glucan.

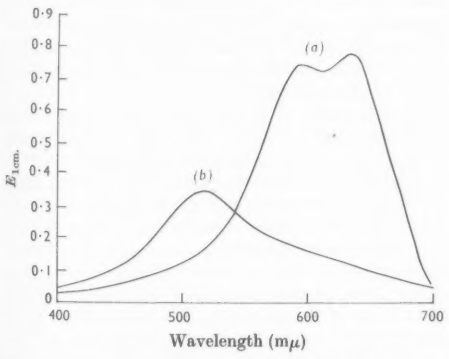

Fig. 4. Absorption spectra of (*a*) free toluidine blue in aqueous solution at 20μM concentration and (*b*) the metachromatic complex, in aqueous solution, of toluidine blue (20μM) and the glucan sulphate (60μM with respect to sulphate).

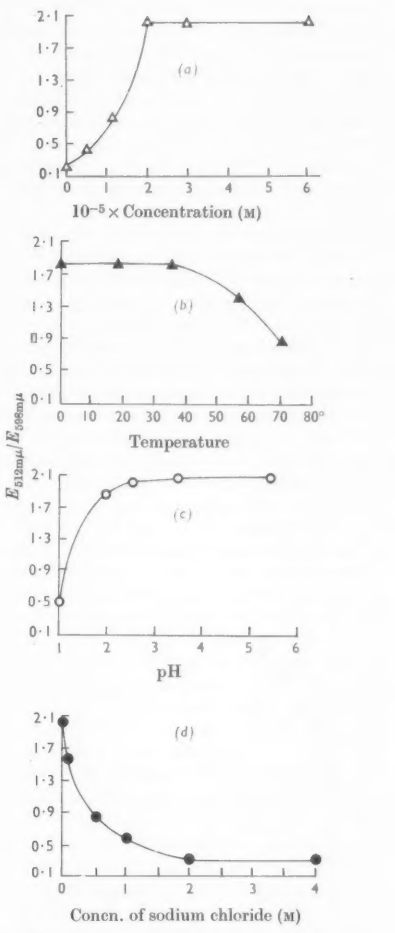

Fig. 5. (*a*) Proportion of metachromatic complex present, at constant dye concentration (20μM-toluidine blue), in aqueous solutions of glucan sulphate at various concentrations with respect to sulphate. (*b*), (*c*) and (*d*) Stability, in aqueous solution, of the metachromatic complex of toluidine blue (20μM) and the glucan sulphate (60μM with respect to sulphate) to increasing temperature (▲), to decreasing pH (○) and to increasing ionic strength (●).

(iv) Metachromasia. The dependence of metachromasia upon concentration of the polysaccharide, upon pH, temperature and ionic strength of the solvent were investigated for the glucan sulphate. The material readily formed a complex with toluidine blue, the absorption spectrum of which showed one maximum at 512mμ, in contrast with the spectrum of the free dye which had maxima at 598 and 653mμ (Fig. 4). An isosbestic point, for the spectra of the complex and free dye, occurs at 545mμ, indicating an equilibrium between the metachromatic and unbound forms of the dye.

The concentration of metachromatic complex present, at constant dye concentration in solutions of varying glucan sulphate concentration, is expressed in Fig. 5 as the E_{512}/E_{598} ratio, as is also the stability of the complex (at the optimum dye and glucan sulphate concentration) to temperature, pH and ionic strength.

Glucan sulphate as the main viscous component of the mucin. A solution of the mucin (0·1%, w/v) in water was adjusted to pH 7·0 with sodium hydrogen carbonate. An appropriate quantity of the proteolytic enzyme Pronase P (Kaken Chemical Co., Tokyo, Japan) was added (1mg. of Pronase P/50mg. of substrate), the solution rapidly mixed and a sample placed in a viscometer maintained at 37°. The remainder of the solution was maintained also at 37° and samples were withdrawn, for degree of proteolysis assay by ninhydrin (Moore & Stein, 1948), at regular intervals over a 24hr. period. The viscosity of the solution was also measured at intervals over the same period (Fig. 6). It can be seen that during removal of the protein by proteolysis the viscosity fell by a negligible amount only.

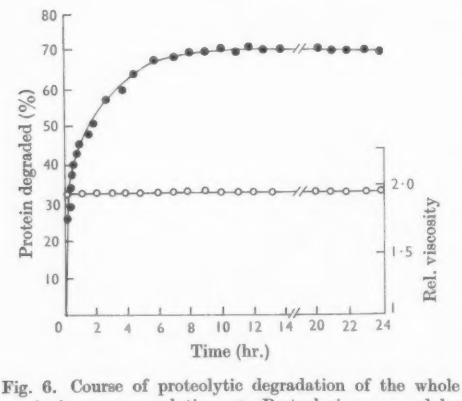

Fig. 6. Course of proteolytic degradation of the whole mucin in aqueous solution. ●, Proteolysis measured by reaction with ninhydrin and expressed as percentage of the total degradation obtained by hydrolysis, of an equal quantity of mucin, in 5·7N-hydrochloric acid at 100° for 16hr. ○, Viscosity of the mucin solution relative to water at the same temperature (37°).

DISCUSSION

Homogeneity. The results obtained from analytical ultracentrifugation and potassium chloride precipitation suggest that the polysaccharide sulphate obtained from the *B. undatum* mucin is a homogeneous preparation.

General composition. The infrared spectra of the material (Fig. 2) indicate that it consists of the major carbohydrate component of the whole mucin and that it is the carrier of the ester sulphate groups detected in the whole mucin (Hunt & Jevons, 1963, 1965*a*), these conclusions being derived from observation of the characteristic carbohydrate bands at 900–1100cm.$^{-1}$ and ester sulphate bands at about 820 and 1250cm.$^{-1}$ (Barker, Bourne & Whiffen, 1956). Peptide bond frequencies were absent, in keeping with the low percentage of peptide detected chemically. The small proportion of peptide material remaining attached to the polysaccharide sulphate is apparently an integral, covalently bound, component and is discussed by Hunt & Jevons (1965*b*). The value 30% for the sulphate (SO_4^{2-}) composition of the material when corrected for peptide and moisture content becomes 35%, which, assuming that the polysaccharide is a glucan, suggests that the ester sulphate residues may be distributed one per monosaccharide unit.

Structure of the polysaccharide. From its monosaccharide composition the polysaccharide can only be a glucan.

Examination of the infrared spectrum of the glucan indicates a strong absorption at 895cm.$^{-1}$ and the absence of absorptions at 844 and 766cm.$^{-1}$, suggesting, on the basis of the criteria of Barker, Bourne, Stacey & Whiffen (1953, 1954) and Barker, Bourne, Weigel & Whiffen (1956), that the linkages might be β rather than α.

The enzymic degradation and partial-acid-hydrolysis data require care in interpretation. Both results tend to suggest the presence of β-(1→4)-linkages, indicating that the glucan has a structure similar to that of cellulose. Moreover the solubility and iodine-staining properties of the glucan are again both similar to those of cellulose. However, with the enzymic evidence a similar effect might have been produced if a second enzyme, specific for β-(1→3)-linkages, were present and if the glucan had a similar structure to lichenin, which contains 30% of β-(1→3)-glucosidic linkages in addition to β-(1→4)-linkages. Dr K. Selby has informed us that the presence of such an enzyme, in the particular culture filtrate used here, cannot be completely discounted. It should, however, be

noted when considering this possibility that the culture filtrate was without action against laminarin, which also has β-(1→3)-linkages. Thus while the results point to some if not all β-(1→4)-structure the possibility of other types of linkage and of a low degree of branching cannot be ruled out.

Position of the sulphate groups. (i) Infrared spectra. The results obtained by infrared spectroscopy are inconclusive. Since all the hydroxyl groups of β-D-glucose are known to be equatorial (Reeves, 1951) it is to be expected that the infrared spectrum should show the characteristic C–O–S frequency (Thompson & Torkington, 1945; Colthup, 1950; Orr, Harris & Sylven, 1952; Orr, 1954) at about 820 cm.$^{-1}$ rather than at 855 cm.$^{-1}$. The broadness of the absorption band (Fig. 2), however, renders difficult any differentiation between primary and secondary substituted equatorial hydroxyl groups although the position of the maximum at 820 cm.$^{-1}$ tends to favour the primary (i.e. the 6-position) (Lloyd & Dodgson, 1961). The inference might be drawn that a proportion of both types is substituted were it not for the information derived from other sources.

With regard to the other frequencies correlated with the presence of sulphate groups (other than at 1240 cm.$^{-1}$), these do not as yet provide information about the position of the groups but are of interest since such absorptions have not previously been widely reported. That the frequencies (940, 920 and 1000 cm.$^{-1}$) are in fact manifestations of the ester sulphate groups, seems to be definitely established by their disappearance on desulphation. A number of workers have reported the presence of absorption frequencies from 920 to 955 cm.$^{-1}$ in the spectra of polysaccharide sulphates but without positively identifying them with the sulphate groups. For example, absorptions at 928 cm.$^{-1}$ in the spectrum of chondroitin sulphate A (Mathews, 1958), at 955 cm.$^{-1}$ in the spectrum of a new chondroitin sulphate (Bettelheim & Philpott, 1960) and at 925 cm.$^{-1}$ for both λ- and κ-carrageenins (Bayley, 1955) have been noted but their disappearance on desulphation has not, however, been recorded. Mathews (1958) has also reported a 1000 cm.$^{-1}$ absorption in chondroitin sulphate and its disappearance on desulphation was reported by Hoffman, Linker & Meyer (1958).

(ii) Rate of hydrolysis of sulphate. The figure of 0·41 hr. for the half-time of the sulphate-release reaction on dilute acid hydrolysis is comparable with that obtained by Rees (1963) for the release of sulphate from β-D-glucose 3-sulphate (i.e. a secondary equatorial hydroxyl position). The release of sulphate from the primary equatorial hydroxyl group at position 6 should, according to Rees (1963), have a half-time of the order 1·5–2·4 hr. The observation that the rate curve was linear suggests strongly that one class of ester sulphate group only is present.

(iii) K^+ ion packing. The property of the material to form an insoluble potassium 'salt', in the presence of potassium chloride, suggests an arrangement of the sulphate on the polysaccharide chain which allows approach and packing together of adjacent chains to produce a highly ordered stack of polymer units in which the 'cement' is provided by hydrated K^+ ions. The diameter of the hydrated K^+ ion is of the order 2·5–4·0 Å (Moelwyn-Hughes, 1940) and, according to Bayley (1955), the optimum distance between alternate sulphate groups attached to adjacent polysaccharide chains, such that entry of a hydrated K^+ ion might be permitted and packing produced, would be of the order 6·0 Å. Examination of molecular models has shown here that with sulphated β-(1→4)-glucans, irrespective of whether the chain is substituted entirely in the 2-, the 3- or the 6-position, the ester sulphate groups will lie at intervals of approximately 12·0 Å and hence when adjacent chains are packed an interval of the order 6·0 Å recurs and K^+ ion stabilization and precipitation could result. Unless all groups are similarly placed, entirely on the 6-, the 3- or the 2-position, this situation would not be permitted. It seems likely therefore that only one type and position of sulphate group on the glucan is possible. This is in keeping with the kinetics of release of sulphate by hydrolysis. Exactly why the precipitation should require a low temperature is not clear.

(iv) Metachromasia. It is apparent from the results that even where the concentration of the polysaccharide is in excess (60 μM with respect to sulphate) over toluidine blue, the bound toluidine blue is all in the metachromatic state, implying that the nearest neighbour binding site must be preferred and that random interactions are at a minimum (Schoenberg & Moore, 1964). The results show clearly that the metachromatic complex has considerable stability even at low pH, high temperature and high ionic strength.

The formation of these highly stable metachromatic complexes by the glucan sulphate would seem to indicate that the polysaccharide molecule possesses a linear configuration upon which the ester sulphate residues are arranged in a precise and regular, rather than random, manner (the considerable stability of the complex might stand as evidence against any significant degree of branching in the polysaccharide chain). This arrangement would agree with the potassium packing property. According to Schoenberg & Moore (1964), for the formation of a metachromatic complex with toluidine blue the acidic residues of a polysaccharide should be capable of approach to about 7 Å separation. Examination of molecular models has

indicated that with a β-(1→4)-glucan chain occupying the normal unstrained configuration, substitution of the 2-, 3- or 6-position by ester sulphate will not fulfil the requirements. If, however, a certain degree of rotation about the glycosidic bond is postulated (the possibility of free rotation about the glycosidic oxygen bond in cellulose is recognized; Woods, 1959), then it becomes possible for ester sulphate residues upon positions 2 or 3, but not on position 6 of the glucose molecules, to approach within the required limits without too great a degree of rotation and strain.

The evidence therefore seems to be weighted slightly against position 6 as the site for the ester sulphate residues on the glucan chain.

Viscosity. From Fig. 6 it can be seen that a 70% degradation of the protein component of the whole mucin, brought about by proteolytic enzyme action, produces no significant change in the viscous properties of the mucin. It seems apparent therefore that the glucan sulphate alone is responsible for the viscosity of the mucin in solution. This situation is comparable with that of the complex of hyaluronic acid and protein occurring in synovial fluid. Here 65% degradation of the protein, by trypsin and chymotrypsin, was achieved without any reduction in the high and anomalous viscosity of the solution occurring (Ogston & Sherman, 1959). It should be borne in mind, however, that the viscosity measurements upon the *Buccinum undatum* mucin were made with dispersions (Hunt & Jevons, 1963) of the original secretion and that these solutions do not possess all of the original rheological properties, i.e. thixotropy and syneresis, in which the protein might well be implicated.

In contrast with a value of the order 5500ml./g. for the intrinsic viscosity of native hyaluronic acid in 0·2M-sodium chloride (Blumberg & Ogston, 1957), that of the glucan sulphate in 0·5M-sodium chloride was of the order 500ml./g. This value should be regarded only as an approximation, as the extrapolation to zero concentration, even in the presence of swamping electrolyte, becomes extremely difficult with the wide scatter of the reduced viscosity values obtained at very high dilutions of polyelectrolytes (see the review by Yang, 1961). The low value for the intrinsic viscosity of the glucan sulphate in comparison with that of hyaluronic acid is most probably a function of the chain length. Calculation of the chain length of the glucan sulphate from the molecular weight $1{\cdot}7 \times 10^5$ and by the use of 10·3Å for the length of the diglucose unit (cellobiose, according to Meyer & Misch, 1937) yields a value of the order of 3200–3300Å. Hyaluronic acid has a chain length of 25000Å (Schubert, 1964) and is thus capable of spreading throughout a large volume of space such that considerable interactions between molecules are possible even in very dilute solution and hence result in elevated viscosities. At equivalent concentrations solutions of the shorter, more compact, sulphate molecules would be expected to produce less extensive interactions than would occur in solutions of the larger, more diffuse, hyaluronic acid molecules.

Comparison with related substances. The value $1{\cdot}7 \times 10^5$ (number average) obtained for the molecular weight of the glucan sulphate is apparently lower than the average for this type of molluscan material. Although no direct data for the molecular weight of the β-(1→4)-linked glucan sulphate in *Charonia lampas* are available, a quoted value of 15s (Iida, 1963), for the sedimentation coefficient, suggests that the molecular weight might be somewhat greater than that of *B. undatum* glucan sulphate (9·75s at infinite dilution). The molecular weight, based on sedimentation data, of the α-(1→6)-glucan sulphate from *C. lampas* mucin was estimated by Iida (1963) as being of the order of 10^6 (estimations of reducing power suggested a value of 10^4). The estimated molecular weight of the polyhexosamine sulphate of *Busycon canaliculatum* mucin, based on light-scattering data (weight average) was $2{\cdot}5 \times 10^6$ (Kwart & Shashoua, 1958). In contrast, chondroitin sulphate, a polysaccharide sulphate of higher animal tissue, has been variously estimated as having chain weights of $2{\cdot}8 \times 10^4$ to $5{\cdot}0 \times 10^4$ (Partridge, 1961; Mathews & Lozaityte, 1956).

With regard to the chemical character, the observation might be made that whereas in higher animals viscous, epithelial secretions consist largely of proteins bearing oligosaccharide side chains with strongly acidic terminal residues in the shape of the sialic acids (Bettelheim-Jevons, 1958; Stacey & Barker, 1962), the corresponding secretions of these lower animals would appear to have achieved the same effect by substitution of linear high-molecular-weight polysaccharides with strongly acidic sulphate groups. The reasons underlying this divergence may reside in the ready availability of sulphate in the marine environment, and here the ubiquity of polysaccharide sulphates in the marine algae (Dillon, 1954) is worthy of note.

S. H. is indebted to the Department of Scientific and Industrial Research for a grant during the period of this work.

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Interferon Production in Chick-Embryo Cells

THE EFFECT OF PUROMYCIN AND *p*-FLUOROPHENYLALANINE

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(*Received* 26 *July* 1965)

1. When chick-embryo cells were treated with ultraviolet-inactivated influenza virus (Melbourne strain), interferon was produced after a lag period of about 10hr. 2. The addition of small amounts of either puromycin or *p*-fluorophenylalanine immediately after the virus inhibited the subsequent production of interferon. Both inhibitors primarily affected protein synthesis, and it is concluded that interferon production involves new protein synthesis. 3. Results obtained by the addition of either inhibitor for short periods during the lag phase demonstrated a requirement for protein synthesis during the second half of the lag phase. 4. Addition of puromycin during the course of interferon production caused almost immediate inhibition, but interferon formation became insusceptible to the action of *p*-fluorophenylalanine at about 26hr. after infection. Possible explanations of this effect are discussed.

Interferon is an antiviral protein produced in a wide variety of animal cells in response to treatment with many viruses (Isaacs, 1963). Its production is inhibited by treatment of the cells with actinomycin, and it has been suggested that it is formed by a process involving de-repression of the host genome (Heller, 1963; Wagner, 1963). Its action is also susceptible to the effect of actinomycin and it may inhibit viral multiplication by a de-repression process (Taylor, 1965). The mechanism of production and action are of interest, not only because of its antiviral activity, but also because its production is an example of the formation of an induced protein in animal cells which can be studied in a tissue-culture system.

We have been studying the formation of interferon in chick-embryo cells after treatment with ultraviolet-inactivated influenza virus (Melbourne strain). The use of an inactivated virus, rather than an infective virus, has advantages since some of the effects due to concomitant virus growth can be avoided, and interpretation of the results is simplified. Intracellular interferon can first be detected about 10hr. after treatment with inactivated virus. The interferon is rapidly released from the cells, and the titre in the medium increases almost linearly with time for about 40hr., after which the rate of production gradually decreases (Burke & Buchan, 1965). The inhibition of interferon production by actinomycin (Heller, 1963; Wagner, 1963) suggests that the synthesis of interferon is controlled by the host rather than by the viral genome. It is not known whether interferon is synthesized anew, or whether an alternative mechanism is involved, e.g. the transformation of an inactive precursor. We have therefore examined the effect of two widely studied inhibitors of protein synthesis, puromycin and FPA,† on the production of interferon. These two inhibitors were chosen because they are known to differ in their mode of action, puromycin causing premature release from the ribosomes of incomplete polypeptide chains (Allen & Zamecnik, 1962), and FPA causing its effect by incorporation into protein in place of phenylalanine, sometimes yielding an inactive protein (Munier & Cohen, 1959; Richmond, 1960, 1963). We have also investigated the effect of addition of these two inhibitors for short periods both before and after the commencement of interferon production in an attempt to obtain information about the intracellular processes on which interferon production is dependent. A brief report of this work has already been presented (Buchan & Burke, 1965).

MATERIALS AND METHODS

Media, cells and viruses. Primary chick-embryo cells were prepared and cultured in medium 199 with added calf

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† Abbreviations: FPA, *p*-fluorophenylalanine; PDD_{50}, 50% plaque-depressing dose; UV-MEL, Melbourne strain of ultraviolet-inactivated influenza virus.

serum as described by Burke & Buchan (1965), growth medium containing 7% of calf serum and maintenance medium 2% of calf serum. The calf serum used in experiments with puromycin or FPA was passed through a Sephadex G-50 column (Piez, Oyama, Levintow & Eagle, 1960) to remove free amino acids and other materials of low molecular weight. Influenza virus (strain Melbourne) was grown in the allantoic cavity of 10-day-old fertile eggs, and inactivated with ultraviolet light as described by Burke (1961). Haemagglutinin titrations were carried out as described by Lindenmann, Burke & Isaacs (1957), the haemagglutinin titre being defined as the reciprocal of that dilution giving partial agglutination. Chikungunya virus was grown in the brains of 3-day-old sucking mice. All viruses were stored at −70°.

Production of interferon. Interferon production was initiated in chick-embryo cells by treatment with UV-MEL as described by Burke & Buchan (1965). The experiments were carried out with two batches of UV-MEL, both of which produced the maximum yield of interferon when a haemagglutinin titre of 50 or higher was used. All experiments were carried out with UV-MEL of haemagglutinin titre 100, to ensure that all cultures received sufficient inactivated virus to produce maximum yields. Three Petri-dish cultures were used for each experimental point, and the fluids pooled and stored at −20° until assayed.

Interferon assay. Interferon was assayed by a plaque-inhibition technique by using Chikungunya virus to challenge (Burke & Buchan, 1965). Samples were diluted in twofold steps, with three dilutions per assay, and incubated with Melbourne virus antiserum before addition in duplicate to chick-embryo cultures. All assays also included six control cultures and suitable dilutions of an internal standard (see below). After overnight incubation at 37°, the cultures were challenged with 50–100 plaque-forming units of Chikungunya virus, and overlaid after 3 hr. Plaques were counted after 3 days, the means of duplicates taken, and the counts, expressed as percentages of the mean control count, plotted against the logarithm of the dilution. The relationship was linear when the plaque count fell between 20 and 80% of the controls, and slope of the dose–response curve was constant, both within any one experiment and also from experiment to experiment. The interferon titre was defined as the reciprocal of the dilution necessary to give a 50% depression of the control plaque count (PDD_{50}, plaque-depressing dose; Lindenmann & Gifford, 1963). All assays contained three dilutions of an internal interferon standard as a check on the reproducibility of the results. The standard had a titre of 63 ± 14 in 22 successive assays over a period of 6 months. Each sample was assayed at least twice, and the mean taken of the titres, which differed by less than 20%.

Incorporation of radioactive precursors. Duplicate cultures in glass Petri dishes were harvested at various times after addition of the radioactive precursor, and the cells washed three times with 3 ml. of cold 0·9% NaCl, three times with 3 ml. of cold 5% (w/v) trichloroacetic acid, twice with 3 ml. of cold ethanol, twice with 3 ml. of ethanol–ether (3:1, v/v) at room temperature and finally with 3 ml. of ether at room temperature. The defatted residue was dissolved by warming to 60° in 3 ml. of 98–100% formic acid, to which 6 drops of 100-volume H_2O_2 had been added, plated at infinite thinness on 2·5 cm.-diam. stainless-steel planchets, and counted by using a thin end-window Geiger–Müller tube, at 3% efficiency, for times sufficient to reduce counting error to less than 5%. The results were corrected for background counting rate. Samples from the 'pulse' experiment with [^{14}C]valine were counted by dissolving the defatted residue in 2 ml. of 0·5 N-NaOH and adding 0·5–10 ml. of a dioxan-based scintillator before counting in the Packard Tri-Carb scintillation counter at 79% efficiency. Less than 10% of the radioactivity from [^{14}C]-valine was found in the fraction soluble in hot 5% (w/v) trichloroacetic acid. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Materials. Racemic FPA was purchased from Calbiochem, Los Angeles, Calif., U.S.A. An aqueous solution (600 μg./ml.) was sterilized by filtration through a Selas porcelain filter candle of maximum pore diameter 1·2 μ and stored at −20°. Puromycin was purchased as the dihydrochloride from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and sterilized and stored in the same way in FPA. Phenylalanine-free medium 199 was a gift from Glaxo Laboratories Ltd., Greenford, Middlesex. L-[U-^{14}C]Valine (specific activity 4·8 mc/m-mole) and [2-^{14}C]uridine (specific activity 36·9 mc/m-mole) were purchased from The Radiochemical Centre, Amersham, Bucks., and *p*-fluoro-DL-3-phenyl[1-^{14}C]alanine (specific activity 3·2 mc/m-mole) was obtained from Calbiochem.

RESULTS

Effect of puromycin on interferon production. The effect of various concentrations of puromycin on the production of interferon is shown in Table 1. Increasing concentrations of puromycin resulted in decreasing interferon yields, a concentration of 1·1 μg./ml. (2 μM) completely inhibiting interferon production. This concentration, which was used in all subsequent experiments, depressed the rate of protein synthesis, as measured by [^{14}C]valine incorporation, to 41% of that of controls. Puromycin has also been shown to inhibit RNA synthesis (Tamaoki & Mueller, 1963; Holland, 1963), although this effect was secondary to that on protein synthesis. The dose of puromycin used only depressed RNA synthesis, as measured by [^{14}C]uridine incorporation, by 10%. It is concluded that the primary

Table 1. *Dose of puromycin required to inhibit interferon production*

After treatment of chick cell monolayers with UV-MEL, 2 ml. of maintenance medium, containing various concentrations of puromycin, was added. At various times triplicate samples were pooled, dialysed against 0·9% NaCl and medium 199 to remove puromycin, and assayed by the plaque-inhibition method.

Concn. of puromycin (μM)	Interferon yield (PDD_{50})		
	At 14 hr.	At 24 hr.	At 36 hr.
0	10	144	309
1	3	42	178
2	<1	<1	<1

effect of this dose of puromycin is on protein synthesis, and that the inhibition of interferon production is probably due to the effect of puromycin on protein synthesis.

The effect of the addition of puromycin for short periods in the lag phase was next examined, to investigate whether interferon production was dependent on protein synthesis during the lag phase. After treatment of cells with UV-MEL, they were incubated for 2, 4, 6 or 8hr. with medium containing puromycin, which was then replaced by normal maintenance medium. Interferon production was followed over the subsequent 36hr. with the results shown in Fig. 1. The presence of puromycin during the initial 4hr. of the lag period did not delay the appearance of interferon (Fig. 1*a*), since the two curves are clearly identical. A similar result was obtained when puromycin was present for the initial 2hr. and then removed. However, when puromycin was present for 6 or 8hr. after infection, delays of about 6 and 8hr. respectively were observed in the time required for interferon formation (Figs. 1*b* and 1*c*), although the rate of interferon production was similar in control and puromycin-treated cultures. These results show that interferon synthesis was more sensitive to puromycin added in the late, rather than the early, part of the lag phase. Since the rate of interferon production resumed at control rates after removal of puromycin, it was decided to determine the effect of preincubation with puromycin, followed by its removal, on [^{14}C]valine incorporation. Cultures were incubated for various times with 2μM-puromycin, the medium was replaced by normal maintenance medium, without washing, and the rate of valine incorporation was measured. Even after 3hr. incubation, the rate of valine incorporation was only 82% of that of controls and the rate was depressed to 56 and 52% of that of controls after 6 or 9hr. incubation respectively with puromycin. Interferon synthesis therefore appears to be more sensitive to the action of puromycin than total cellular protein synthesis, and to resume more rapidly after removal of puromycin inhibition.

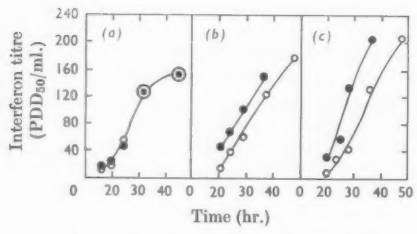

Fig. 1. Effect of the presence of puromycin (2μM) for (*a*) 4hr., (*b*) 6hr. and (*c*) 8hr. after treatment of chick cells with UV-MEL, on the subsequent production of interferon (○), as compared with controls (●). Experimental details are given in the Materials and Methods section.

The effect of the addition of puromycin during the later stages of interferon production was next investigated. At intervals after the stimulation of interferon production, the medium was removed and replaced by pre-warmed maintenance medium containing puromycin. Control cultures received similar treatment with maintenance medium. After 48hr. the fluids were harvested and dialysed before assaying for interferon, with the results shown in Table 2. The interferon titres in the control column show the interferon yield between the time stated and 48hr. after infection, and decrease as the time-interval decreases. Comparison with the values in the third column, which shows the interferon yield in puromycin-treated cultures, indicated that puromycin was most effective in suppressing interferon formation if added during the first 12hr. after infection, but that only small and constant amounts of interferon were formed after the addition of the inhibitor when it was added later in the cycle. The effect of the inhibitor was not due to inhibition of interferon release since the intracellular interferon titres in inhibited cultures at 58hr. after infection were identical with those found in control cultures. Since washing of the cultures during the production of interferon was found to decrease the final yield of interferon, it was avoided. To keep disturbance of the cells to a minimum, this experiment was also carried out by adding puromycin directly to the cultures and assaying for interferon yield at 39hr. after infection. The results (Table 3) show that addition of the antibiotic caused immediate inhibition of interferon synthesis. The slightly lower

Table 2. *Effect of puromycin on interferon production*

Medium was removed from the cultures and replaced by medium containing puromycin (2μM) at different times throughout the production cycle of interferon. The fluids were collected at 48hr., and dialysed before assay for the interferon formed between the time of addition of the inhibitor and 48hr. after infection.

Time of addition of inhibitor (hr. after infection)	Interferon yield at 48hr. (PDD_{50})	
	Control	Inhibitor
0	135	<1
12	78	<1
17	—	8
22	59	13
26	—	14
30	—	14
36	30	11

Table 3. *Addition of puromycin during interferon production*

Puromycin (0·2 ml. of 20 μM) was added to chick cultures at various times after treatment with UV-MEL, and incubation continued until 39 hr. after infection. The fluids were then dialysed and assayed for interferon. Control cultures were harvested at the time at which the inhibitor was added.

Time of addition of inhibitor (hr. after infection)	Interferon yield (PDD_{50})	
	In control cultures at time of addition of inhibitor	In treated cultures at 39 hr. after infection
12	26	18
15	50	40
18	80	53
24	98	98
39	160	—

Table 4. *Dose of FPA required to inhibit interferon production*

Medium, containing various concentrations of FPA in phenylalanine-free medium, was added to chick-cell cultures immediately after removal of UV-MEL, control cultures being incubated with complete medium 199 (i.e. containing phenylalanine). At intervals, triplicate samples were harvested, dialysed against 0·9% NaCl and medium 199, and assayed for interferon.

Concn. of FPA (μg./ml.)	Interferon yield (PDD_{50})		
	At 12 hr.	At 24 hr.	At 48 hr.
Control	12	214	309
0	<1	128	—
5	—	5	40
25	<1	<1	2
50	—	<1	<1

titres in the treated cultures were probably due to thermal inactivation of interferon.

Effect of FPA on interferon production. To determine the dose of FPA required to inhibit interferon production, chick-cell cultures, infected with UV-MEL, were incubated with 2 ml. of phenylalanine-free medium 199 containing FPA at various concentrations and the yield of interferon was measured at various times. Table 4 shows that a concentration of 25 μg. of FPA/ml. completely depressed interferon yield, and this concentration was used in further studies. The decreased yield in phenylalanine-free medium, as compared with controls, is probably due to exhaustion of the intracellular phenylalanine pool. The action of FPA was not due to a delay in interferon release,

Table 5. *Effect of FPA on* [^{14}C]*valine incorporation into chick cells*

Phenylalanine-free medium, with or without FPA, and [^{14}C]valine (0·1 μc/dish) were added to chick cells at the times shown. Duplicate samples were taken at hourly intervals for 5 hr., over which period incorporation was linear with time. The activity of the control cultures after 5 hr. incubation was 600 counts/min./culture.

Concn. of FPA (μg./ml.)	Time of addition of FPA and [^{14}C]valine (hr. after infection)	Uptake of [^{14}C]valine (% of controls)
0	0	64
25	0	65
25	12	65
25	22	73
25	31	71

since the interferon yields from FPA-treated cultures at 63 and 72 hr. after infection were not significantly greater than those found at 48 hr. after infection. Nor was its inhibitory effect due to an inhibition of interferon release, since only very small amounts of intracellular interferon could by detected in FPA-treated cultures.

Measurement of the effect of FPA on protein synthesis (Table 5) showed that it depressed the rate of valine incorporation, but that this depression was due to the absence of phenylalanine from the medium rather than to the presence of FPA. Other experiments showed that the inhibitory effect was completely and rapidly reversed by the addition of normal medium, and that [^{14}C]FPA was incorporated into cell protein. Incubation of cells with FPA for up to 8 hr. did not depress the rate of incorporation of subsequently added [^{14}C]-uridine into RNA, showing that there was no effect of this dose of FPA on RNA synthesis.

When the cells were incubated with medium containing FPA for 3 hr. after treatment with UV-MEL, and then incubated in normal medium, the rate of subsequent interferon production was depressed, although there was no increase in the lag period (Fig. 2*a*). However, the presence of FPA for 6 hr. prolonged the lag period by several hours, and almost completely inhibited subsequent interferon production (Fig. 2*b*). Since FPA was shown to be incorporated into cell protein, the inhibitory effect of FPA is probably due to the formation of non-functional proteins, normally essential for interferon production.

The effect of the addition of FPA during the later stages of interferon production was also investigated. At intervals after treatment of

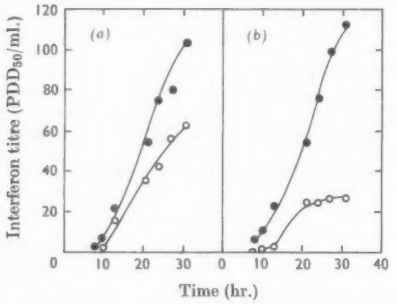

Fig. 2. Effect of the presence of FPA (25 μg./ml.) for (*a*) 3 hr. and (*b*) 6 hr. after treatment with UV-MEL on the subsequent production of interferon (○), as compared with controls (●). Experimental details are given in the Materials and Methods section.

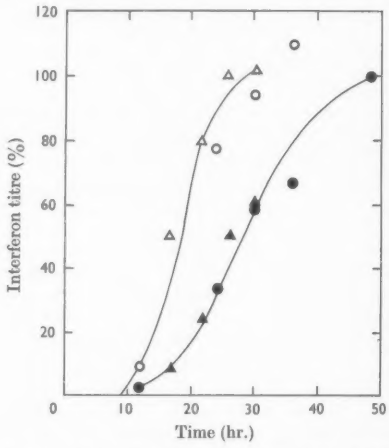

Fig. 3. Effect of the addition of FPA (25 μg./ml. in phenylalanine-free medium) on the subsequent production of interferon. FPA was added at various times and the interferon titre at the time of addition and at 48 hr. after infection determined in the usual way. The left-hand curve represents the sum of the interferon titres before addition of FPA and between addition of FPA and 48 hr. after infection (○ and △), as a percentage of the sum of the corresponding titres in the control cultures. The right-hand curve represents the interferon titre of control cultures as a percentage of the titre at 48 hr. after infection (● and ▲). Two experiments are shown with final control titres at 48 hr. after infection of 380 and 145.

cultures with UV-MEL, the medium was removed and replaced by medium containing FPA. At 48 hr. after infection the fluids were harvested, dialysed and assayed for interferon, along with the fluids harvested before addition of FPA. FPA almost completely inhibited interferon production when added during the first 12 hr. after infection, but its inhibitory effect decreased over the subsequent 14 hr., until at 26 hr. after infection, and later, it produced no depression of subsequent interferon synthesis. Fig. 3 shows the decreasing susceptibility of interferon production as a function of the time of addition of FPA. The interferon yields in the control cultures are also plotted for comparison. The difference between the two curves is a measure of the increasing resistance of interferon production to FPA.

This increasing resistance could have been due to a change in permeability of the cells to FPA. However, it was found that the inhibitory effect of FPA on the rate of incorporation of [^{14}C]valine was constant, whether the inhibitor was added at 12, 22 or 31 hr. after infection (Table 5), i.e. at times when it has a very different effect on interferon synthesis. It was also found that the rate of uptake of [^{14}C]FPA was linear and independent of the time of its addition.

Attempt to measure increased protein synthesis early in interferon production. Since the experiments with puromycin and FPA had shown that protein synthesis during the latter half of the lag period was essential for interferon production, an attempt was made to detect increased protein synthesis in UV-MEL-treated cultures. Incorporation of [^{14}C]valine was measured at consecutive 1 hr. intervals until 8 hr. after infection. It was found that the specific activities of the UV-MEL-treated cultures differed from those of the control cultures by less than 10%, i.e. within the limits of error of the method. Presumably the protein synthesis necessary for interferon production represents only a small fraction of that going on within the cell.

DISCUSSION

These results show that low concentrations of two inhibitors of protein synthesis, puromycin and FPA, inhibit the production of interferon in chick cells. The synthesis of interferon therefore involves new protein synthesis rather than some alternative mechanism, such as transformation of an inactive precursor. A similar conclusion has been reached by Ho & Breinig (1965) as a result of experiments with puromycin.

The presence of puromycin in the medium for the initial 2 or 4 hr. after infection did not increase the length of the lag period, nor inhibit the subsequent rate of interferon production. It appears that interferon synthesis does not require protein synthesis during the 4 hr. immediately after infection.

Zimmerman & Schafer (1960) and Wheelock (1962) have shown that the early stages in the multiplication of fowl-plague virus and Newcastle-disease virus are independent of protein synthesis. The latter author observed that adsorption and penetration of Newcastle-disease virus occurred within 1 min. of infection, and he suggested that the subsequent 2 hr. period, which was insensitive to a dose of puromycin completely blocking all protein synthesis, was required for the removal of protein and lipid from the virus particle. Similar events may be required for interferon formation after adsorption of inactivated influenza virus. When puromycin was present for 6 or 8 hr. after infection, the lag period was increased by 4–6 and 6–8 hr. respectively, but the rates of formation were not significantly different in inhibited and control cultures. These results suggest that protein synthesis is required during the entire initial 6 or 8 hr. periods, and are not consistent with those results obtained when 2 or 4 hr. incubation periods with puromycin were employed. However, removal of puromycin from chick-cell cultures did not result in a complete reversal of the inhibition of protein synthesis, the extent of reversal decreasing with increasing time of incubation with puromycin. Puromycin therefore causes some irreversible damage to the mechanism of protein synthesis, and this is possibly the explanation of the increase of the lag period by more than the expected period of 2 or 4 hr. Mueller, Kajiwara, Stubblefield & Rueckert (1962) have observed a similar effect of puromycin on protein synthesis.

The increase in the length of the lag period when puromycin was present during the first 6 or 8 hr. after infection indicates that protein synthesis is necessary during the latter half of the lag period. This protein may be interferon, although interferon was undetectable within chick cells during the lag period, or it may be an enzyme essential for interferon synthesis. An attempt to detect increased protein synthesis, as compared with controls, during the lag period was unsuccessful. The inhibitory effect produced when puromycin was added during the production of interferon demonstrates a continued requirement for protein synthesis as long as interferon is being produced, the small amounts of interferon formed after the addition of the antibiotic probably being due to the time taken for puromycin to exert its effect.

FPA exerts its inhibitory effect on protein synthesis by a mechanism different from that of puromycin. The minimum concentration of FPA that completely inhibited interferon synthesis (25 μg./ml.) produced only a small inhibition of [^{14}C]valine incorporation, and it was shown that this inhibition was due to the absence of phenylalanine from the medium rather than to the presence of FPA. Use of [^{14}C]FPA showed that the inhibitor was incorporated into cellular protein, presumably with the formation of non-functional proteins (Scholtissek & Rott, 1961). Other workers have needed to use much higher doses of FPA to inhibit virus-induced processes (e.g. Wilcox & Ginsberg, 1963), mainly because of the use of medium containing phenylalanine, which will compete with the inhibitor. We have used phenylalanine-free medium and calf serum from which small molecules had been removed by gel filtration, and this is probably the reason for the effectiveness of the low dose employed. In contrast with puromycin, FPA had no detectable effect on RNA synthesis.

Incubation of the cells with FPA for 3 hr. immediately after treatment with UV-MEL had no effect on the length of the lag period, but depressed the rate of subsequent interferon production. This result suggests that incubation with FPA led to the formation of a non-functional form of a protein normally essential for the synthesis of interferon, and formed within 3 hr. after infection. This protein appears to be different from the one whose synthesis is inhibited by puromycin, since no requirement for protein synthesis could be demonstrated during this period by treatment with the dose of puromycin used. The presence of FPA for 6 hr. prolonged the lag period as well as depressing the final yield of interferon, and the increased lag may be due to an effect on the puromycin-sensitive protein.

When FPA was added immediately after infection with UV-MEL, or up to 12 hr. after infection, it completely inhibited interferon production. Interferon formation then became gradually less susceptible to inhibition, and at 26 hr. after infection no inhibition was detectable. This change in susceptibility was not due to a change in permeability of the cells nor to a change in the size of the phenylalanine pool, since the incorporation of [^{14}C]FPA, and the inhibitory effect of FPA on [^{14}C]valine uptake, were unchanged throughout the production period. Our explanation of this effect is that a second, FPA-sensitive, protein is synthesized until about 26 hr. after infection, and that this protein is stable and essential for interferon production. Certain proteins involved in adenovirus synthesis also display an increasing resistance to the action of FPA (Wilcox & Ginsberg, 1963). Extrapolation of the curves of Fig. 3 suggest that production of this FPA-susceptible protein begins about 8 hr. after infection or a little earlier. This protein may be identical with the puromycin-sensitive protein, first formed about 6 hr. after infection.

The results obtained by the use of these two inhibitors suggest that at least three proteins are

involved in interferon production: an FPA-sensitive protein formed within 3hr. of treatment with UV-MEL, a puromycin-sensitive protein first detectable about 6hr. after infection and possibly identical with a second, FPA-sensitive, protein whose formation is complete by 26hr. after infection, and interferon itself.

We thank the Department of Scientific and Industrial Research, the Medical Research Council, Glaxo Laboratories Ltd., Imperial Chemical Industries Ltd., Pharmaceuticals Division, and the Wellcome Research Laboratories for grants which supported this work, and Mr F. Troup and Mr G. Sutherland for technical assistance.

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[^{35}S]Thiosulphate Oxidation by *Thiobacillus* Strain C

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(*Received* 27 *July* 1965)

1. *Thiobacillus* strain C oxidized [^{35}S]thiosulphate completely to sulphate. 2. During thiosulphate oxidation [^{35}S]sulphate was formed more rapidly from $(S\cdot{}^{35}SO_3)^{2-}$ than from $({}^{35}S\cdot SO_3)^{2-}$. ^{35}S disappeared less rapidly from thiosulphate with $({}^{35}S\cdot SO_3)^{2-}$ as substrate than with $(S\cdot{}^{35}SO_3)^{2-}$. 3. Thiosulphate labelled in both atoms was produced during $({}^{35}S\cdot SO_3)^{2-}$ oxidation, but not during $(S\cdot{}^{35}SO_3)^{2-}$ oxidation. 4. No ^{35}S was precipitated as elementary sulphur either in the presence or absence of exogenous unlabelled sulphur. 5. During [^{35}S]thiosulphate oxidation, appreciable quantities of [^{35}S]trithionate accumulated and later disappeared. Other polythionates did not accumulate consistently. 6. [^{35}S]Trithionate was formed initially at a greater rate from $(S\cdot{}^{35}SO_3)^{2-}$ than from $({}^{35}S\cdot SO_3)^{2-}$, but subsequently at a similar rate from each. 7. Trithionate formed from $(S\cdot{}^{35}SO_3)^{2-}$ was labelled only in the oxidized sulphur atoms, but that formed from $({}^{35}S\cdot SO_3)^{2-}$ was labelled in both oxidized and reduced atoms. The proportion of ^{35}S in the oxidized atoms increased as more trithionate accumulated. 8. The results eliminate some mechanisms of trithionate formation but are consistent both with a mechanism of thiosulphate oxidation based on an initial reductive cleavage of the molecule and with a mechanism in which thiosulphate undergoes an initial oxidative reaction.

Chemoautotrophic bacteria of the genus *Thiobacillus* derive energy for growth from the oxidation of reduced inorganic sulphur compounds, the principal oxidation product being sulphate. Studies of these organisms have led to the postulation of two apparently conflicting mechanisms for the oxidation of thiosulphate to sulphate (Vishniac & Santer, 1957; Vishniac & Trudinger, 1962; Peck, 1962; Kelly, 1965). The first of these mechanisms, a polythionate pathway, involves the initial oxidation of thiosulphate to tetrathionate, and the subsequent oxidation of this to sulphate with the probable intermediate formation of other polythionates and possibly of organic sulphur compounds. London & Rittenberg (1964) and Pankhurst (1964) have presented evidence for such a mechanism, but Trudinger (1964*b*) has suggested the occurrence of an S_4 intermediate, other than tetrathionate, but derived from two thiosulphate ions. Such a compound might be the product *in vivo* of an enzyme that has been shown to form tetrathionate from thiosulphate *in vitro* (Trudinger, 1961; Vishniac & Trudinger, 1962). The second mechanism is based on work with cell-free extracts by Peck (1960) and involves the initial reduction of thiosulphate to sulphide and sulphite. These products are subsequently oxidized to sulphate with the intermediate formation of adenosine 5′-sulphatophosphate but not of polythionates; the sulphite moiety may first be oxidized to thiosulphate.

Clearly, the mechanism of thiosulphate oxidation is complex, and is not yet explained satisfactorily. By using [^{35}S]thiosulphate we have sought to determine (1) whether there is discriminative oxidation of the two sulphur atoms of thiosulphate as expected from the Peck hypothesis (cf. Ostrowski & Krawczyk, 1957; Peck & Fisher, 1962; Trudinger, 1964*b*,*c*), and (2) the origin and function of polythionates in the oxidation. Our results are consistent with an oxidation mechanism based on Peck's hypothesis, but may also be consistent with a modified polythionate oxidation pathway.

MATERIALS AND METHODS

Organism. Thiobacillus strain C was used in all experiments. This organism has been described elsewhere as *T. thioparus* (Kelly & Syrett, 1964*a*,*b*; Kelly, 1965), but may more closely resemble *T. neapolitanus* (D. White & M. Hutchinson, personal communication). It was cultured as

described elsewhere (Kelly & Syrett, 1964*a*). Organisms centrifuged from batch cultures were washed in sodium phosphate buffer, pH 7·0, and resuspended in 0·1 M-Na_2HPO_4–NaH_2PO_4, pH 7·0, to give known cell densities between 0·2 and 2·0 mg. dry wt./ml.

Radioactive thiosulphate. Anhydrous $Na_2(S{\cdot}SO_3)$, labelled in either the 'outer' (S–) or 'inner' (–SO_3) position with ^{35}S, was obtained from The Radiochemical Centre, Amersham, Bucks. The nomenclature adopted in this paper to describe the position of the ^{35}S label is as follows: $Na_2(^{35}S{\cdot}SO_3)$ is described as 'reduced-atom-labelled', and $Na_2(S{\cdot}^{35}SO_3)$ as 'oxidized-atom-labelled'. Similarly, the central sulphur atom of trithionate $(O_3S{\cdot}S{\cdot}SO_3)^{2-}$ is referred to as the 'reduced atom' and the outer two as the 'oxidized atoms'.

Experiments with [^{35}S]thiosulphate. The ^{35}S-labelled $Na_2S_2O_3$ was added to bacteria shaken vigorously in Warburg flasks or conical flasks at constant temperature. In kinetic experiments, bacteria were separated rapidly from the suspending medium by filtration through Oxoid or Millipore membranes, or were killed by pipetting samples of suspensions into equal volumes of ethanol, and subsequently separated by centrifuging. The filtrates and ethanolic supernatants were analysed by paper chromatography.

Oxygen consumption was followed by Warburg manometry at 30°. Analysis of Warburg flask contents was made after separating the solution from the bacteria by filtration or by centrifuging. In some experiments elementary sulphur added to the flasks was largely freed of bacteria by low-speed centrifugation, washed with 50% (v/v) ethanol and acetone, and dried at 60° before assay for ^{35}S.

Chromatography and radioautography. Paper chromatograms were run on Whatman no. 1 paper developed in one dimension with descending solvents at 22 ± 1°. The solvent commonly used was butan-1-ol–acetone–water (2:2:1, by vol.) (Skarzynski & Szczepkowski, 1959), run for 16–20 hr. Other solvents used were pyridine–propan-1-ol–water (3:5:5, by vol.) (Trudinger, 1959) and butan-1-ol–methanol–water (1:1:1, by vol.) (The Radiochemical Centre Data Sheet DS 3552). Marker compounds, $Na_2S_2O_3$, $K_2S_3O_6$ and $K_2S_4O_6$, were detected as Ag_2S on the papers by spraying with 0·5% (w/v) $AgNO_3$ in aq. NH_3 solution [5 vol. of aq. NH_3 (sp.gr. 0·88) + 95 vol. of water) and heating at 100°.

[^{35}S]Sulphate and [^{35}S]thiosulphate were also used as marker materials. [^{35}S]Polythionate markers were produced by incubating [^{35}S]thiosulphate with unlabelled $K_2S_3O_6$ and $K_2S_4O_6$, when distribution of ^{35}S among all three compounds occurred (Fava, 1953).

Radioactive compounds on chromatograms were detected by placing the papers on Kodak Kodirex X-ray film for 2–4 weeks. Radioactive areas were outlined by tracing over the developed film.

To distinguish between [^{35}S]thiosulphate and [^{35}S]sulphate (which have very similar R_F values in the solvents used) samples were treated with excess of iodine; this completely converted thiosulphate into tetrathionate, which was easily separated chromatographically from sulphate. The quantities of sulphate and thiosulphate were calculated from the difference between samples with and without iodine treatment. Sulphate and thiosulphate were also separated by running chromatograms in butan-1-ol–acetone–water for 24–36 hr.

Degradation of radioactive trithionate. [^{35}S]Trithionate was eluted from preparative paper chromatograms, after location by radioautography, and degraded by reaction with $HgCl_2$. Samples (1 or 2 ml.) of aqueous solutions of [^{35}S]trithionate in micro-centrifuge tubes were mixed with 0·5 ml. of 36% (w/v) formaldehyde solution and 0·5 ml. of 5% (w/v) $HgCl_2$ in 2% (w/v) sodium acetate. After the mixture had stood at 22° for 15 min., 2 mg. of unlabelled $K_2S_3O_6$ in 0·1 ml. of water was added, and the mixture was left for a further 1½–2 hr. Like tetrathionate (van der Heijde & Aten, 1952; Trudinger, 1961), trithionate broke down to sulphate and an insoluble mercury complex (Abegg, Auerbach & Koppel, 1927; Starkey, 1935) as follows:

$$2K_2(O_3\overset{*}{S}{\cdot}S{\cdot}\overset{*}{S}O_3) + 3HgCl_2 + 4H_2O \rightarrow$$

$$HgCl_2,2HgS + 2K_2\overset{*}{S}O_4 + 2H_2\overset{*}{S}O_4 + 4HCl$$

Thus the reduced atom of trithionate was precipitated and the oxidized ones remained in solution as sulphate. The precipitate was removed by centrifuging and washed once with a 1:20 dilution of the H·CHO–$HgCl_2$–sodium acetate reagent. The supernatant and washings were made up to a known volume for assay of ^{35}S. The precipitate was oxidized with 1 ml. of conc. HNO_3 saturated with bromine and boiled gently for 30 min. on a sand bath. The solution was made up to standard volume with water.

Degradation of radioactive thiosulphate. Two methods were used.

(*a*) The method used for trithionate (with 4 mg. of $Na_2S_2O_3,5H_2O$ instead of $K_2S_3O_6$ as carrier) converted the oxidized atom of thiosulphate into sulphate, and precipitated the reduced atom as $HgCl_2,2HgS$ (van der Heijde & Aten, 1952), which was oxidized as above.

(*b*) The thiosulphate solution was heated at 95° with excess of $AgNO_3$ for 45 min. The oxidized atom was left in solution as sulphate and the reduced atom precipitated as Ag_2S (Brodskii & Eremenko, 1954). The precipitate was removed by centrifuging and washed with water. The supernatant and washings were made up to standard volume, and the precipitate was either suspended in a standard volume of water or dissolved in 2% (w/v) KCN for assay of ^{35}S.

Assay of ^{35}S. (*a*) Radioactive spots on chromatograms were counted on the paper by using a thin end-window Geiger–Müller tube linked to a decade scaler (Ekco Electronics Ltd.). Counts were corrected for background radiation and, when necessary, for the 'dead time' of the counter. The count obtained from a radioactive spot was proportional to the amount of ^{35}S present (with [^{35}S]sulphate standards), at least over the range 1–200 mμc of ^{35}S/spot. Also, the count given by a radioactive spot was essentially the same from each side of the paper, after the chromatogram had been run and allowed to dry evenly (Kelly, 1965). Normally only one side of the paper was counted.

(*b*) Solutions of ^{35}S compounds were dried on 2·5 cm.-diam. ground-glass disks, usually to give infinitesimally thin samples for counting with a thin end-window Geiger–Müller tube. When thick samples were used, corrections for self-absorption were applied by reference to samples of infinitesimal thinness. Samples were counted to give a counting error not greater than ± 3%.

(*c*) In some experiments with elementary sulphur, weighed quantities of dried sulphur were dissolved in CS_2 and dried on disks as infinitesimally thin samples for count-

ing as in (*b*). In one experiment, sulphur was oxidized with HNO_3–Br_2–HCl (Trudinger, 1961).

Sulphate estimation. Sulphate was determined turbidimetrically as $BaSO_4$ (Gleen & Quastel, 1953). Samples containing up to about 10 μmoles of sulphate were mixed with 2 ml. of 50% (v/v) glycerol and water to a total volume of about 7 ml. Then 1 ml. of 10% (w/v) $BaCl_2$ in 4·8% (v/v, conc. acid in water) HCl was added and the volume immediately made up to 10 ml. with water before mixing very thoroughly. Standards and suitable blanks were prepared and turbidities measured by using a Hilger Spekker absorptiometer with dark-blue filters and 1 cm. cuvettes.

Reagents. AnalaR chemicals were used when available. Other reagents were of the highest purity available commercially. $K_2S_3O_6$ and $K_2S_4O_6$ were a generous gift from Dr F. H. Pollard of Bristol University. Finely divided, easily wettable elementary sulphur was obtained from old cultures of *T. thio-oxidans* and repeatedly washed before drying at 108°.

RESULTS

Oxygen uptake during thiosulphate oxidation. Both dilute bacterial suspensions (0·1–0·5 mg. dry wt./Warburg flask) and dense ones (3–7 mg./flask) consumed oxygen at a high and often constant rate, giving a total uptake usually very close to that calculated for complete thiosulphate oxidation to sulphate (Starkey, 1935):

$$Na_2S_2O_3 + 2O_2 + H_2O \rightarrow Na_2SO_4 + H_2SO_4$$

Dense suspensions did not visibly precipitate elementary sulphur in such experiments (contrast Vishniac & Trudinger, 1962).

Oxidation of $Na_2(^{35}S \cdot SO_3)$ (*i.e. radioisotope in reduced sulphur atom*). After the addition of [^{35}S]-thiosulphate to dilute suspensions, the disappearance of radioactivity from the thiosulphate fraction was accompanied by [^{35}S]sulphate production at an almost equal rate (Fig. 1). The identity of the

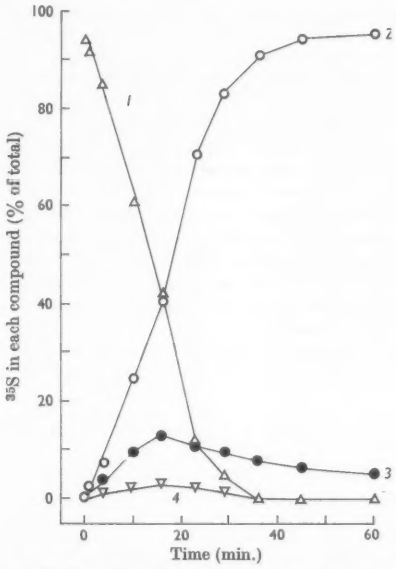


Fig. 1. Oxidation of $Na_2(^{35}S \cdot SO_3)$ by *Thiobacillus* strain C. Experimental details are given in Table 1. [^{35}S]Thiosulphate was added at zero time. Curve *1*, thiosulphate; curve *2*, sulphate formation; curve *3*, trithionate; curve *4*, tetrathionate.

Table 1. *Specific activity of* [^{35}S]*sulphate produced during the oxidation of* $Na_2(^{35}S \cdot SO_3)$

Thiobacillus strain C (10 mg. dry wt.) was shaken at 30° in a final volume of 48 ml. of 0·1 M-sodium phosphate buffer, pH 7·0, in a 500 ml. conical flask; 196 μmoles of $Na_2(^{35}S \cdot SO_3)$ (144 μc) were added and 5 ml. samples filtered through membranes at intervals. Filtrates were analysed by paper chromatography (Fig. 1) and the total sulphate content was determined. No loss of ^{35}S accompanied filtration, indicating that no elementary [^{35}S]sulphur was precipitated. The added thiosulphate contained 331·8 counts/sec./μmole. If the two sulphur atoms of thiosulphate were converted into sulphate at equal rates, the specific activity of sulphate at any sampling time would be 165·9 counts/sec./μmole.

Sample time (min.)	Sulphate/ml. of filtrate (μmoles)	[^{35}S]Sulphate/ml. (counts/sec.)	Specific activity (counts/sec./ μmole)	Specific activity (% of theoretical)
0	0	0	—	—
4	1·4	94·6	67·7	41
10	3·5	338·0	96·8	58
16	5·5	541·0	98·6	59
23	7·0	960·0	137·2	83
29	7·8	1122·0	144·0	87
36	8·0	1228·0	154·0	93
45	8·0	1271·0	159·0	96
60	8·2	1282·0	156·7	94
Calc. maximum	8·17	1355·4	165·9	100

sulphate was confirmed by chromatography in three solvents, and by its precipitation with 97–100% efficiency by acid barium chloride. A lag of 0·5–2 min. preceded the establishment of a high constant rate of [^{35}S]sulphate production (Figs. 1 and 4). A substance chromatographically indistinguishable from trithionate increased during thiosulphate disappearance, and decreased as thiosulphate became exhausted. With a dense cell suspension, similar but more rapid kinetics were obtained. Comparison of total sulphate and [^{35}S]-sulphate production (Table 1) showed that the specific activity of the sulphate was initially less than half the expected value, but increased as more sulphate was formed. This result would occur if the unlabelled oxidized sulphur atom of thiosulphate were converted into sulphate faster than the labelled reduced one. The final discrepancy in specific activity would be explained if the trithionate remaining after thiosulphate exhaustion (and accounting for about 5% of the total ^{35}S) were derived largely from the reduced atom of thiosulphate.

Accumulation of trithionate. [^{35}S]Trithionate accumulated consistently in all experiments (Figs. 1 and 2). Its persistence after thiosulphate exhaustion is doubtless related to its lower rate of oxidation by the bacteria, the Q_{O_2} for thiosulphate oxidation being 1130 ± 280 μl. of oxygen/hr./mg. dry wt. (mean ± S.D. of 27 values) compared with 430 ± 200 (mean ± S.D. of 5 values) for trithionate. When a dense cell suspension was used thiosulphate disappeared completely within 5 min. After its disappearance the trithionate that had accumulated was oxidized completely to sulphate at a rate significantly lower than the original rate of sulphate production from thiosulphate.

Accumulation of tetrathionate. Usually the accumulation of [^{35}S]tetrathionate and [^{35}S]pentathionate during $Na_2(^{35}S \cdot SO_3)$ oxidation accounted for less than 3% of the total ^{35}S. However, in one exceptional experiment, a transitory accumulation of a large amount of [^{35}S]tetrathionate was observed. Its formation was accompanied by a sudden fall of the amount of thiosulphate.

Comparison of oxidation of $Na_2(^{35}S \cdot SO_3)$ *and* $Na_2(S \cdot {}^{35}SO_3)$. Fig. 2 shows that [^{35}S]sulphate production and [^{35}S]thiosulphate disappearance were more rapid from $(S \cdot {}^{35}SO_3)^{2-}$ than from $(^{35}S \cdot SO_3)^{2-}$. Degradation of thiosulphate in the 20 sec. and 60 sec. samples from the $Na_2(^{35}S \cdot SO_3)$ series showed that 1–2% and 5–7% respectively of the ^{35}S in the thiosulphate was in the oxidized atom. [^{35}S]Trithionate was produced at a similar rate from both substrates, although it was produced faster initially from $Na_2(S \cdot {}^{35}SO_3)$. These results were supported by another experiment, in which doubly-labelled thiosulphate, $Na_2(^{35}S \cdot {}^{35}SO_3)$, was also supplied (Table 2).

Degradation of labelled trithionate. Table 3 shows the distribution of ^{35}S in the trithionate formed. Trithionate formed from $Na_2(S \cdot {}^{35}SO_3)$ was labelled only in the oxidized atoms, whereas that from $Na_2(^{35}S \cdot SO_3)$ contained a progressively larger proportion of ^{35}S in the oxidized atoms, reaching a maximum of about 50% of the total ^{35}S in trithionate. The proportion in the oxidized atoms may have continued to increase slightly after exhaustion of the thiosulphate substrate. Similarly, trithionate formed from $Na_2(S \cdot {}^{35}SO_3)$ in the experiment of Table 2 showed no significant labelling of the reduced atom, whereas that derived from $Na(^{35}S \cdot SO_3)$ after 300 and 420 sec. contained 28 and 31% respectively of its total ^{35}S in the oxidized atoms. The addition of unlabelled $K_2S_3O_6$ to

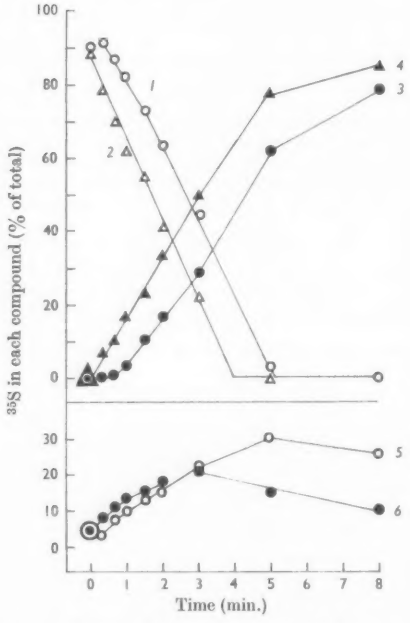


Fig. 2. Oxidation of $Na_2(^{35}S \cdot SO_3)$ or $Na_2(S \cdot {}^{35}SO_3)$ by *Thiobacillus* strain C. The 250 ml. flasks shaken at 23° contained (in a final volume of 9 ml.) 700 μmoles of sodium phosphate buffer, pH 7·0, and 14 mg. dry wt. of bacteria; 42 μmoles of $Na_2S_2O_3$ (containing 15 μc of ^{35}S in the reduced atom or 7 μc of ^{35}S in the oxidized atom) were added at zero time. Samples (1 ml.) were pipetted into 1 ml. of ethanol. Curves *1*, *3* and *5*: thiosulphate, sulphate and trithionate respectively from $(^{35}S \cdot SO_3)^{2-}$; curves *2*, *4* and *6*: thiosulphate, sulphate and trithionate respectively from $(S \cdot {}^{35}SO_3)^{2-}$.

Table 2. *Oxidation of thiosulphate labelled in either or both positions with* ^{35}S*: the formation of* [^{35}S]*sulphate and* [^{35}S]*trithionate*

Thiobacillus strain C (1·85 mg./ml.) in 14 ml. of 0·1 M-sodium phosphate buffer, pH 7·0, shaken at 23° in 250 ml. conical flasks, received 2 ml. of [^{35}S]thiosulphate (100 μmoles) containing 30 μc of ^{35}S in the oxidized or reduced atom or 30 μc in each atom. Samples (2 ml.) were pipetted into 2 ml. of ethanol at 0° at intervals, centrifuged and the supernatants analysed chromatographically. In all experiments, 95–103% of the added ^{35}S was recovered in $S_2O_3^{2-}+SO_4^{2-}+S_3O_6^{2-}+S_4O_6^{2-}+S_5O_6^{2-}$.

Substrate ...	^{35}S in each compound (% of ^{35}S added)								
	... $Na_2(^{35}S\cdot SO_3)$			$Na_2(S\cdot{}^{35}SO_3)$			$Na_2(^{35}S\cdot{}^{35}SO_3)$		
Time (sec.)	$S_2O_3^{2-}$	SO_4^{2-}	$S_3O_6^{2-}$	$S_2O_3^{2-}$	SO_4^{2-}	$S_3O_6^{2-}$	$S_2O_3^{2-}$	SO_4^{2-}	$S_3O_6^{2-}$
0	100	—	—	100	—	—	100	—	—
30	92	5·8	0·4	91	6·0	2·0	94	6·6	1·0
55	90	7·0	1·3	85	9·5	1·7	89	8·8	1·1
90	77	8·0	4·0	74	13·0	4·2	80	11·7	2·7
125	75	9·0	4·7	69	17·0	4·7	70	15·0	5·1
180	69	14·0	5·4	59	22·0	8·8	64	21·9	7·1
300	47	29·0	15·1	29	47·5	17·0	42	40·9	17·7
420	9	45·0	32·0	7	68·5	22·0	11	56·0	28·1

Table 3. *Distribution of* ^{35}S *in trithionate formed from thiosulphate labelled in the reduced or oxidized atom*

Expt. I. Details were as given for Fig. 2. Samples (1 ml.) were pipetted into an equal volume of ethanol and centrifuged, and trithionate in the supernatants was separated chromatographically for degradation. Expt. II. A 250 ml. conical flask shaken at 26° contained (in a final volume of 12 ml.) 33 mg. dry wt. of *Thiobacillus* strain C and 1 m-mole of sodium phosphate buffer, pH 7·1; 47 μmoles (23 μc) of $Na_2(^{35}S\cdot SO_3)$ were added at zero time.

Substrate ...		... $Na_2(^{35}S\cdot SO_3)$			$Na_2(S\cdot{}^{35}SO_3)$		
		Distribution (% of recovered ^{35}S)		^{35}S recovery in assay	Distribution (% of recovered ^{35}S)		^{35}S recovery in assay
	Sample time (sec.)	–S–	$-SO_3^-$	(%)	–S–	$-SO_3^-$	(%)
Expt. I	20	80·0	20·0	94·1	0	100·0	104·2
	60	77·9	22·1	96·1	0	100·0	95·5
	90	70·9	29·1	93·0	—	—	—
	120	68·8	31·2	98·7	1·0	99·0	98·6
	180	59·3	40·7	98·2	—	—	—
	300	48·2	51·8	104·0	1·6	98·4	95·5
	480	44·3	55·7	105·0	1·3	98·7	103·2
Expt. II	420	46·7	53·3	112·0	—	—	—
	600	52·0	48·0	98·5	—	—	—

oxidized-atom-labelled trithionate before degradation did not decrease the proportion of ^{35}S in the oxidized sulphur atoms. Spontaneous [^{35}S]trithionate formation occurs in mixtures of [^{35}S]-thiosulphate and unlabelled trithionate but at a rate that is much too low to account for its formation in our experiments (Fava, 1953; D. P. Kelly, unpublished work).

Use of a 'trap' of elementary sulphur to detect free sulphur formation from the reduced atom of thiosulphate. In the absence of added elementary sulphur no significant loss of ^{35}S from solution was ever demonstrated when the reaction mixtures were either membrane-filtered or centrifuged (contrast Trudinger, 1964*b*,*c*). Fig. 3 shows the oxygen consumption of dilute cell suspensions of *Thiobacillus* strain C oxidizing $Na_2(^{35}S\cdot SO_3)$ in the absence or presence of a large 'pool' of finely divided elementary sulphur, which also served as a substrate for oxidation. Table 4 demonstrates that, after the cessation of rapid oxidation, no ^{35}S had been retained in the sulphur 'trap'. This result contrasts with that of Peck & Fisher (1962). Chromatography showed 96–100% of the added ^{35}S to be present as

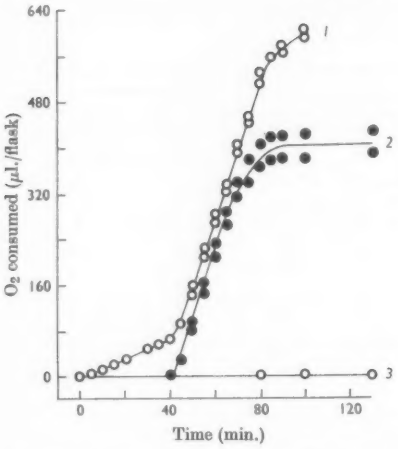


Fig. 3. Simultaneous oxidation of $Na_2(^{35}S \cdot SO_3)$ and elementary sulphur by *Thiobacillus* strain C. Experimental details were as given in Table 4. Thiosulphate was added at 40 min. Curve *1*, with sulphur; curve *2*, without sulphur; curve *3*, boiled bacteria, with or without sulphur.

Table 4. [^{35}S]*Thiosulphate oxidation in the presence of a 'trap' of elementary sulphur*

Warburg flasks contained (in a final volume of 2·4 ml.) 0·5 mg. dry wt. of living or dead (boiled for 2 min.) *Thiobacillus* strain C and 200 μmoles of sodium phosphate buffer, pH 7·0, with or without 100 mg. of fine elementary sulphur, and shaken at 30°. After preincubation for 40–50 min. 9·8 μmoles (7·2 μc) of $Na_2(^{35}S \cdot SO_3)$ were added. Oxidation is shown in Fig. 3. After the final manometric reading, the flask contents were filtered through membranes and the ^{35}S content of filtrates and residues was determined. Washed membranes + sulphur were first oxidized with Br_2–HNO_3–HCl. Average values for duplicates are given.

Treatment	$10^{-4} \times$ ^{35}S (counts/100 sec./flask)		Recovery of added ^{35}S in filtrate (%)
	Filtrate	Sulphur residue	
Initial thiosulphate	5·437	0	100·0
Live cells—sulphur	5·374	0	98·5
Live cells+sulphur	5·500	0	101·0
Dead cells—sulphur	5·160	0	95·0
Dead cells+sulphur	5·042	0	93·0

sulphate in the flasks containing living cells. In a second experiment, dense or dilute bacterial suspensions oxidized $Na_2(^{35}S \cdot SO_3)$ with a sulphur pool (Fig. 4) and total analyses of ^{35}S distribution in the reaction mixtures were made both during

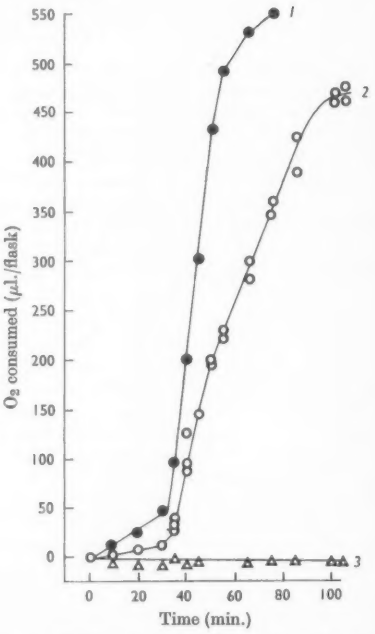


Fig. 4. Simultaneous oxidation of $Na_2(^{35}S \cdot SO_3)$ and elementary sulphur by dense and dilute suspensions of *Thiobacillus* strain C. Experimental details were as given in Table 5. Thiosulphate was added at 30 min. Curve *1*, dense suspensions; curve *2*, dilute suspensions; curve *3*, boiled bacteria, both densities.

rapid oxidation and immediately after its cessation (Table 5). No [^{35}S]sulphur was formed, but with the dilute suspensions labelled trithionate accumulated as usual and ^{35}S was present in the oxidized atoms of trithionate and thiosulphate, just as it was in the absence of elementary sulphur.

DISCUSSION

Since no elementary [^{35}S]sulphur was precipitated during [^{35}S]thiosulphate oxidation nor was any trapped in a pool of added sulphur, either sulphur is not an intermediate in the oxidation of thiosulphate by *Thiobacillus* strain C or, if it is, it failed to exchange at all with exogenous sulphur even though this was being oxidized simultaneously.

A preferential formation of sulphate from the oxidized sulphur atom of thiosulphate (Table 2 and Fig. 2) has also been observed by Trudinger (1964c) with *Thiobacillus* X and by Smith (1965) with *Chromatium*. This finding is consistent with

Table 5. *Oxidation of* $(^{35}S \cdot SO_3)^{2-}$ *and the formation of* [^{35}S]*trithionate in the presence of a pool of elementary sulphur*

Warburg flasks contained (in a final volume of 2·9ml.) 0·4mg. dry wt. (dilute) or 4·0mg. dry wt. (dense) of *Thiobacillus* strain C, 200μmoles of sodium phosphate buffer, pH7·0, and 40mg. of elementary sulphur; after preincubation at 30°, 10·9μmoles (4μc) of $Na_2(^{35}S \cdot SO_3)$ were added and oxidation was followed manometrically (Fig. 4). Duplicate flasks were removed during (dilute) and at the end of (dilute and dense) rapid oxidation and the sulphur was separated by centrifugation, washed, dried and dissolved in CS_2. The supernatants were mixed with ethanol for chromatography, and sampled for ^{35}S content. Thiosulphate and trithionate were purified from mixtures for degradation. Average values for duplicates are given.

(*a*) Distribution of ^{35}S in total samples

Treatment	Sample time (see Fig. 4) (min.)	$10^{-3} \times$ ^{35}S (counts/100sec./flask)		Recovery of added ^{35}S (%)	Distribution of ^{35}S in supernatants (%)			
		Supernatant	S^0 pool		$S_2O_3^{2-}$	SO_4^{2-}	$S_3O_6^{2-}$	$S_4O_6^{2-}$
Boiled (dense)	80	282	0	99·0	84·7	3·1	5·5	6·9
Living (dense)	80	299	0	105·0	0	99·4	0·6	0
Boiled (dilute)	105	260	0	91·0	86·1	1·5	5·5	6·9
Living (dilute)	54	277	0	98·0	46·9	27·0	20·4	5·8
Living (dilute)	105	286	0	99·0	0	81·5	18·5	0

(*b*) Distribution of ^{35}S in the isolated thiosulphate and trithionate

Treatment	Compound degraded	Distribution (% of recovered ^{35}S)		^{35}S recovery in assay (%)
		Reduced atom	Oxidized atom	
Boiled (dilute) 105min.	Thiosulphate	92·2	7·8	89·6
	Trithionate	81·0	19·0	83·3
Living (dilute) 54min.	Thiosulphate	63·9	36·1	100·3
	Trithionate	51·3	48·7	89·3

a number of different possible mechanisms of thiosulphate oxidation. In the mechanism proposed by Peck (1960, 1962), thiosulphate undergoes an initial reductive cleavage by which the oxidized sulphur atom appears as sulphite and is oxidized to sulphate by two further reactions, whereas the reduced atom appears as sulphide whose oxidation pathway is unknown. Similarly, by any mechanism of thiosulphate metabolism in which the first step is its oxidation to tetrathionate or a related S_4 compound, it seems clear that more reactions will be required to oxidize the reduced sulphur atom to sulphate than to complete the oxidation of the oxidized sulphur atom, which is already combined with three oxygen atoms. Thus, on either hypothesis, preferential formation of sulphate from the inner, oxidized sulphur atom of thiosulphate might be expected.

When thiosulphate was supplied as $(S \cdot ^{35}SO_3)^{2-}$ rather than as $(^{35}S \cdot SO_3)^{2-}$, not only was [^{35}S]sulphate formed more rapidly but [^{35}S]thiosulphate disappeared more quickly. It is not at once obvious why this should be so, since any reaction of thiosulphate must result in the disappearance of the whole molecule. A possible explanation comes from the observation that thiosulphate labelled in both sulphur atoms appeared within 20sec. when $(^{35}S \cdot SO_3)^{2-}$ was oxidized. This suggests that, after an initial reaction, the reduced sulphur atom of $(^{35}S \cdot SO_3)^{2-}$ is oxidized, forming uniformly labelled thiosulphate. Thus the lag in the disappearance of [^{35}S]thiosulphate when $(^{35}S \cdot SO_3)^{2-}$ is the substrate would be due to the resynthesis of labelled thiosulphate. Indeed, from Fig. 2, it can be deduced that between the fourth and fifth minutes of this experiment the thiosulphate remaining in solution must have been derived almost entirely from the reduced sulphur atom of the initial thiosulphate, since during the first 4min. all the radioactivity supplied as $(S \cdot ^{35}SO_3)^{2-}$ had been converted into sulphate or trithionate. If the initial reaction of thiosulphate is its reductive cleavage to sulphide and sulphite, uniformly labelled thiosulphate could arise by the oxidation of the sulphide moiety to thiosulphate, as Peck & Fisher (1962) have suggested. On the other hand, it could arise from the two central atoms of a tetrathionate molecule formed by the initial oxidation of thiosulphate.

As Trudinger (1964c) has pointed out, polythionate formation during thiosulphate oxidation by thiobacilli is variable and dependent on a number of factors including the way in which the organisms were grown. Thus not too much significance should be attached to the accumulation of trithionate,

rather than other polythionates, in our experiments. Nevertheless an analysis of the course of [^{35}S]trithionate production from [^{35}S]thiosulphate throws some light on the mechanism of thiosulphate oxidation.

During the first 30 sec. of thiosulphate oxidation, [^{35}S]trithionate was formed more quickly from $(S\cdot{}^{35}SO_3)^{2-}$ than from $(^{35}S\cdot SO_3)^{2-}$ (Table 2). Thereafter the rate of [^{35}S]trithionate formation was the same from either substrate. These facts rule out the possibility that trithionate was formed by the simple oxidation of tetrathionate:

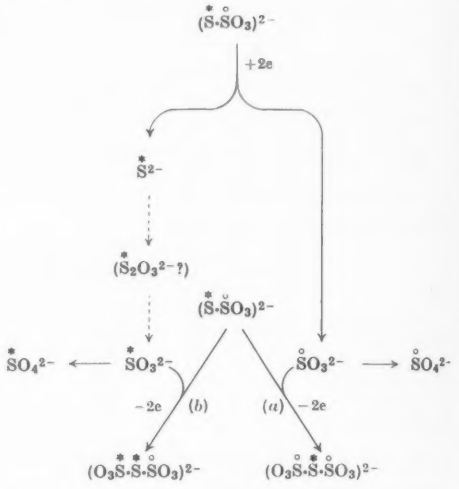
$$\underset{\text{thiosulphate}}{2(\overset{*}{S}\cdot\overset{\circ}{S}O_3)^{2-}} \xrightarrow{-2e} \underset{\text{tetrathionate}}{(O_3\overset{\circ}{S}\cdot\overset{*}{S}\cdot\overset{*}{S}\cdot\overset{\circ}{S}O_3)^{2-}} \xrightarrow[+3O]{+2e} \overset{\circ}{S}O_3^{2-} + \underset{\text{trithionate}}{(O_3\overset{*}{S}\cdot\overset{*}{S}\cdot\overset{\circ}{S}O_3)^{2-}}$$

because, by this mechanism, the rate of ^{35}S appearance in trithionate would be twice as rapid when ^{35}S was supplied in the reduced sulphur atom of thiosulphate rather than in the oxidized one.

Moreover, such a mechanism fails to explain the distribution of ^{35}S within the trithionate molecules. The results show that, when $(^{35}S\cdot SO_3)^{2-}$ is supplied, the first-formed trithionate is labelled predominantly in the central, reduced sulphur atom, but as oxidation proceeds the proportion of ^{35}S in the outer, oxidized atoms of trithionate increases and, when thiosulphate oxidation ceases, the two oxidized atoms together contain about half of the total ^{35}S in the trithionate (Table 3).

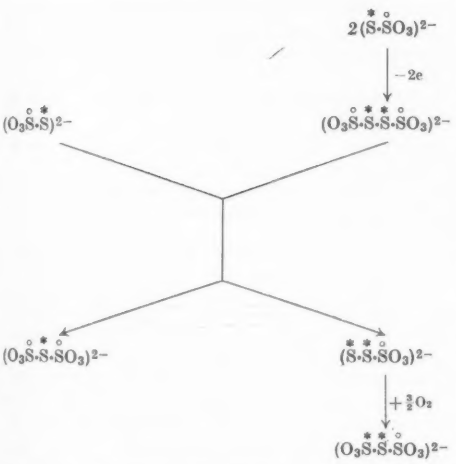
These results can be accounted for in at least two different ways. First, they are consistent with the hypothesis of Peck (1960) provided that the reduced sulphur atom of thiosulphate, which, by his mechanism, appears first as sulphide, is oxidized to sulphate with sulphite as an intermediate. Evidence that suggests that sulphite is formed from both atoms of thiosulphate has, in fact, been presented by Santer (1959) and by Peck & Stulberg (1962) from ^{18}O-labelling experiments. Trithionate might then arise by an enzyme-catalysed oxidative reaction between thiosulphate and sulphite (Scheme 1). By this mechanism one would expect that, at first, trithionate would be formed by reaction (*a*), since sulphite would be produced more quickly initially from the oxidized sulphur atom of thiosulphate; this initial trithionate would be labelled only in the central sulphur atom when $(^{35}S\cdot SO_3)^{2-}$ was the substrate. As the reduced sulphur atom of thiosulphate also appeared as sulphite, reaction (*b*) would take place and, when both reactions (*a*) and (*b*) were equally rapid, the rate of incorporation of ^{35}S into trithionate would be the same whether the substrate was $(^{35}S\cdot SO_3)^{2-}$ or $(S\cdot{}^{35}SO_3)^{2-}$. Moreover, ^{35}S from $(^{35}S\cdot SO_3)^{2-}$ would appear in increasing amounts in the oxidized sulphur atoms of trithionate and one would expect, finally, two-thirds of the ^{35}S in trithionate to be in the reduced central atom and one-third in the oxidized atoms. However, when $(^{35}S\cdot SO_3)^{2-}$ was supplied, thiosulphate labelled in both sulphur atoms appeared (Table 5 and Trudinger, 1964*c*). If this doubly labelled thiosulphate were to participate in the reactions postulated in Scheme 1, a greater proportion of ^{35}S would be introduced into the oxidized sulphur atoms of trithionate when $(^{35}S\cdot SO_3)^{2-}$ was the original substrate. A more equal distribution of ^{35}S between the reduced and oxidized sulphur atoms of trithionate would then be expected, in agreement with the observed result (Table 3). One would expect, too, that [^{35}S]trithionate formation from $(S\cdot{}^{35}SO_3)^{2-}$ would terminate before that from $(^{35}S\cdot SO_3)^{2-}$, as Fig. 2 shows. Thus the mechanism of Scheme 1 is consistent with our results. On this view trithionate arises, by oxidation, as a by-product of the main sequence of thiosulphate oxidation. On the Peck mechanism, the initial attack of thiosulphate is reductive. Both tetrathionate formation and trithionate formation from thiosulphate are oxidative processes; their function may be to provide electrons for the reductive cleavage of thiosulphate. The subsequent oxidation of the polythionates might involve their prior reduction to thiosulphate and sulphite (Imai, Okuzumi & Katagiri, 1962; Trudinger, 1964*a*).

Other mechanisms of trithionate formation are, however, possible and a second way in which our



Scheme 1. Hypothetical reactions explaining trithionate formation.

results could be accounted for is by a reaction between thiosulphate and tetrathionate, giving trithionate as a product. Pankhurst (1964) suggests that trithionate is formed like this by a purely chemical, i.e. non-enzyme-catalysed, reaction. However, experiments have shown that the rate of formation of labelled trithionate when [^{35}S]thiosulphate and tetrathionate are shaken together is far too low to account for [^{35}S]trithionate formation in our cultures (D. P. Kelly, unpublished work). Moreover, the amount of tetrathionate present at any time is very small. Nevertheless, trithionate might be formed by an enzyme-catalysed reaction between thiosulphate and tetrathionate and, if a series of reactions like those of Scheme 2 took place, both the rate of formation of [^{35}S]trithionate from $(^{35}S \cdot SO_3)^{2-}$ or $(S \cdot {}^{35}SO_3)^{2-}$ and the distribution of ^{35}S within the trithionate molecules would be the same as those predicted by the mechanism of Scheme 1. Thus our results, though eliminating some mechanisms of trithionate formation, do not distinguish unequivocally between mechanisms of thiosulphate oxidation based on an initial reductive attack, as in Scheme 1, and mechanisms based on an initial oxidation, as in Scheme 2.



Scheme 2. Alternative hypothetical reactions explaining trithionate formation.

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The Concentration and Biosynthesis of Nicotinamide Nucleotides in the Livers of Rats Treated with Carcinogens

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(*Received* 30 *June* 1965)

1. The oxidoreduction state and concentration of both NAD and NADP as well as the maximum potential activities of NMN adenylyltransferase and NAD^+ kinase have been measured in the livers of rats treated for 14–28 days with 4-dimethylamino-3′-methylazobenzene, 4-dimethylamino-4′-fluoroazobenzene, α-naphthyl isothiocyanate or ethionine and in primary hepatomas induced by 4-dimethylamino-3′-methylazobenzene. 2. The total NAD and total NADP both decreased in the livers of rats treated with either azo-dyes or α-naphthyl isothiocyanate but not in those treated with ethionine. The activities of NMN adenylyltransferase and NAD^+ kinase did not alter appreciably after such treatments. 3. In the primary hepatomas the concentrations of both NAD and NADP fell drastically and the activities of NMN adenylyltransferase and NAD^+ kinase fell to about 50% of the control activities. 4. No correlation could be established between the concentrations of the nucleotides and the activities of the enzymes synthesizing them. It appears, however, that a relationship exists between the NAD content of the tissue and the amount of NADP present. 5. The results are discussed with respect to the control of NAD and NADP synthesis by ATP. At the concentrations of NAD normally present in the cell it is suggested that NAD may be a rate-limiting substrate in NADP synthesis.

Morton (1958) suggested that an inverse relationship exists between the rate of cell division in a tissue and the NAD content of that tissue, a generalization that has an obvious bearing on studies of tissues, such as tumours and foetal cells, where the rate of division is high. On this basis, one of the prerequisites for the initiation of rapid cell division in the precancerous state would be a decrease in the concentration of the nucleotide. Although there have been many studies of the concentration of NAD in tumour material, there have been relatively few in the early stages of carcinogenesis. Notable among the latter are the results of Jedeikin, Thomas & Weinhouse (1956), which showed a tendency for the NAD concentration in the precancerous liver to decrease with increasing time of treatment with the carcinogen 3′MeDAB,† and also those of Nodes & Reid (1964), who found that azo-dye carcinogenesis produced little change in NAD concentration in the early stages but tended to lower the NADP content. The decline of NAD content is even more marked in well-developed tumours and has been adequately documented for a number of tissues and a variety of carcinogens (Jedeikin & Weinhouse, 1955; Glock & McLean, 1957; Briggs, 1960; Wintzerith, Klein, Mandel & Mandel, 1961; Missale & Colajacomo, 1963; Nodes & Reid, 1964). The NADP content of hepatomas is also much lower than that of normal liver (Glock & McLean, 1957; Briggs, 1960; see Reid, 1962).

In the light of these observations it appeared worth while to study the oxidized and reduced forms of NAD and NADP in both the precancerous phase, after feeding with a number of agents with different potencies in respect to their ability to produce malignant change, and in primary hepatomas produced by one of these agents. The activities of two of the enzymes involved in the synthesis of these nucleotides, namely NMN adenylyltransferase (EC 2.7.7.1) and NAD^+ kinase

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† Abbreviations: 3′MeDAB, 4-dimethylamino-3′-methylazobenzene; 4′FDAB, 4-dimethylamino-4′-fluoroazobenzene.

(EC 2.7.1.23), have also been measured in view of the above-mentioned changes in concentration and the observation by Quastel & Zatman (1953) that the NAD^+-glycohydrolase activity of tumours was within the range found in normal tissue and hence could not have contributed to the depletion of these nucleotides. Measurements on NMN adenylyltranferase are pertinent in view of the results of Branster & Morton (1956), who found that this enzyme was less active in mammary tumours of mice than in the normal gland, and of the suggestion by Morton (1961) of a possible role for NMN adenylyltransferase as a regulator of tissue NAD content and of cell division.

METHODS

Animals. Unless otherwise stated, male albino rats, aged about 7 weeks and with initial body weights of about 200g., were used.

Treatment of rats with azo-dyes

Precancerous livers. The precancerous state was induced by the feeding of two azo-dyes (3′MeDAB and 4′FDAB) that are known to be hepatocarcinogens. The azo-dyes were supplied to the rats in the diet, which contained 20% of protein and 0·075% of azo-dye, for periods varying from 8 to 42 days. The diet was given to the experimental animals in weighed amounts and control rats were given the same weight of a similar diet except that it contained no carcinogen. On the day before they were killed, the rats were put into individual cages and given the 20%-protein diet, without dye, in restricted amount such that the rats were being starved at the time they were killed.

Treatment of rats with α-naphthyl isothiocyanate. To provide a comparison, some rats were treated with α-naphthyl isothiocyanate, which is said to be non-carcinogenic but which produces cell proliferation of the bile duct (Grant & Rees, 1958). This was also supplied in the same diet and at the same concentration as the azo-dyes, and the treatment of rats before they were killed was as described for those given azo-dyes.

Hepatomas. The rats were given a diet containing 20% of protein and 0·075% of 3′MeDAB for about 100 days. They were then given the stock diet *ad lib.* until hepatomas developed, usually between 7 and 12 months from the beginning of feeding with the azo-dye. Controls for these rats were taken as those of the same age that had been fed on the 20%-protein stock diet throughout the time.

Treatment of rats with ethionine

Female albino rats, body weight approx. 150g., were given a diet compounded of powdered rat cube diet 41B (Bruce & Parkes, 1949) and ethionine in aqueous solution so as to produce a paste in which the DL-ethionine concentration was 0·25%. This diet was fed *ad lib.* to the treated animals, while control animals were pair-fed with these experimental animals by using a similar diet but containing no ethionine.

Extraction and estimation of the nicotinamide nucleotides

Extraction procedures. The oxidized forms of the nucleotides were extracted in 0·1 N-HCl and the reduced forms in 0·1 N-NaOH according to the method of Glock & McLean (1955). A separate extraction in 0·02 N-H_2SO_4–0·1 M-Na_2SO_4, by the method of Burch, Lowry & Von Dippe (1963), was made by using rapidly frozen tissue as described by these authors, so that the 'extra' NADP reported by them could be measured. All the nicotinamide nucleotides were assayed by the polarographic method of Greenbaum, Clark & McLean (1965*a*).

Estimation of enzyme activities. NMN adenylyltransferase and NAD^+ kinase were estimated as described by Greenbaum, Clark & McLean (1965*c*).

Chemicals

3′MeDAB, 4′FDAB and α-naphthyl isothiocyanate were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. All other chemicals used were as described by Greenbaum *et al.* (1965*a*,*c*) or were of AnalaR grade.

Expression of results and statistical treatment

Reid (1962) has examined critically the various bases on which results obtained from tumour tissues may be expressed and has concluded that the least objectionable parameter is per g. of tissue. For this reason, all the results given below are expressed either as μg. of nucleotide/g. wet wt. of tissue or as μg. of NAD or NADP formed/g. wet wt. of tissue/hr. For the statistical evaluation of results the 'paired-*t* test' has been used.

RESULTS

Precancerous livers produced by azo-dyes

Concentration of the nicotinamide–adenine dinucleotides. Rats were treated with either 3′MeDAB or 4′FDAB for times varying between 8 and 42 days and the concentration of nicotinamide nucleotides in the liver was examined. There appeared to be no discernible trend over this time with either of the carcinogens, and accordingly only data for the largest groups, i.e. those treated for 14–28 days, have been included in Table 1. The NAD^+ concentration was decreased by both carcinogens. NADH was not greatly affected by 3′MeDAB, but 4′FDAB decreased the concentration to about 45% of that in normal liver. 4′FDAB also caused a significant fall in the $NADP^+$ concentration, although 3′MeDAB failed to do so. Both carcinogens decreased NADPH concentration. It is noteworthy that α-naphthyl isothiocyanate caused a substantial rise in the concentration of $NADP^+$. The large variations among animals were not statistically significant, but nevertheless some of the highest concentrations of $NADP^+$ reported in liver were found in certain of these animals. The

range of values was 7–122μg./g. with four values greater than the average for the control group. Our results show rather larger falls in the concentration of the nucleotides for both azo-dyes than those given in the literature (Jedeikin *et al.* 1956; Kotnis, Narurkar & Sahasrabudhe, 1962; see Reid, 1962).

Activities of NMN adenylyltransferase and of NAD$^+$ kinase. The effects of the same compounds on the activities of NMN adenylyltransferase and of NAD$^+$ kinase are shown in Table 2. In Table 2 are also included the sums of NAD$^+$+NADH concentrations and NADP$^+$+NADPH concentrations for comparison with the enzyme activities. The activity of NMN adenylyltransferase is significantly decreased by 4′FDAB but 3′MeDAB has little effect. This difference is in accord with the greater effect of the fluoro compound in decreasing the amount of NAD (Table 2, column 4). Neither carcinogen decreased the activity of the NAD$^+$ kinase, which is somewhat surprising in view of the fact that the same dose of carcinogen for the same length of time decreased the amount of NADP in the tissue.

Hepatomas produced by azo-dyes

Concentration of the nicotinamide–adenine dinucleotides. Only hepatomas derived from rats fed with 3′MeDAB for long periods were used in this investigation and the results for these are shown in Table 3. The concentrations of all four forms of the nucleotide are dramatically decreased. The decreases for these are: NAD$^+$, 70%; NADH, 80%; NADP$^+$, 65%; NADPH, 85%; ($\sum$NAD, 74%; $\sum$NADP, 85%). These findings confirm published data on nucleotide concentrations in tumours (Glock & McLean, 1957; Briggs, 1960; Wintzerith *et al.* 1961; see Reid, 1962). Glock & McLean (1957) noted that, although the concentrations of NAD and NADP were decreased, there was no difference in the ratio of the oxidized to reduced forms in either case. The present results confirm this for NAD but, as there was a greater proportional loss of the reduced form for NADP, there was a shift in the oxidized/reduced ratio for this coenzyme. Nodes & Reid (1964) have also found an increased NADP$^+$/NADPH ratio.

Activities of NMN adenylyltransferase and NAD$^+$ kinase. The effect of long-term treatment with 3′MeDAB on the activity of these two enzymes is shown in Table 4, which, again, includes the sums of NAD$^+$+NADH concentrations and NADP$^+$+NADPH concentrations for comparison. The activities of both these enzymes fall to about 50% of the levels in normal liver. A similar fall in the activity of NMN adenylyltransferase was recorded by Branster & Morton (1956) in mammary tumours of mice and by Atkinson, Jackson & Morton (1961) in nuclei isolated from hepatomas of pig liver.

Table 1. *Oxidoreduction state and concentration of the nicotinamide nucleotides in the livers of rats treated with azo-dyes or with α-naphthyl isothiocyanate*

The nicotinamide nucleotides were extracted and assayed by the method of Greenbaum *et al.* (1965*a*). The results are expressed as means ± S.E.M. Fisher's P in parentheses represents the probability of difference between the means. Values of Fisher's P greater than 0·1 are shown as N.S., i.e. not statistically significant. The diet containing the compounds at a concentration of 0·75% was fed to the rats for 14–28 days.

Compound given	No. of animals	Body wt. (g.)	Liver wt. (g.)	Conen. (μg./g. wet wt.) NAD$^+$	NADH	$\frac{\text{NAD}^+}{\text{NADH}}$ ratio	NADP$^+$	NADPH	$\frac{\text{NADPH}}{\text{NADP}^+}$ ratio
None	7	297±26	11·9±0·2	380·0±41	120·0±21	3·79±0·71	20·6±3·8	267·0±28	14·2±1·4
3′MeDAB	8	278±16	12·5±0·73	246·0±38 (0·016)	95·3±13·2 (0·1)	3·43±0·8 (N.S.)	17·7±3·3 (N.S.)	176·5±14·2 (0·002)	15·2±3·3 (N.S.)
4′FDAB	8	269±13	10·3±1·0	230·0±23 (0·001)	53·6±7·1 (0·0002)	5·76±0·99 (0·09)	15·0±2·1 (0·04)	153·5±23 (0·004)	13·0±3·0 (N.S.)
α-Naphthyl isothiocyanate	6	269±13·6	13·8±1·0	272·5±42 (0·05)	114·6±16·9 (N.S.)	3·07±0·66 (N.S.)	45·7±16·2 (N.S.)	166·0±23·8 (0·008)	8·69±3·59 (N.S.)

Table 2. *Total cellular concentrations of NAD and NADP and the activities of NMN adenylyltransferase and NAD+ kinase in the livers of rats treated with azo-dyes or with α-naphthyl isothiocyanate*

The nicotinamide nucleotides and enzymes were assayed by the methods of Greenbaum *et al.* (1965*a*,*c*). The results are expressed as means ± S.E.M. Other conditions were as given in Table 1.

Compound given	No. of animals	Body wt. (g.)	Liver wt. (g.)	ΣNAD (μg./g.)	NMN adenylyltransferase (μg. of NAD formed/g./hr.)	ΣNADP (μg./g.)	NAD+ kinase (μg. of NADP formed/g./hr.)
None	7	297±26·0	11·9±0·2	506±55	3755±368	287±31	1991±175
3′MeDAB	8	278±16·0	12·5±0·73	370±16 (0·001)	3467±506 (N.S.)	221±13 (0·001)	1865±182 (N.S.)
4′FDAB	8	269±13·0	10·3±1·0	301±12 (0·001)	2885±405 (0·02)	161±25 (0·004)	2020±229 (N.S.)
α-Naphthyl isothiocyanate	6	269±13·6	13·8±1·0	370±41 (0·051)	3364±350 (N.S.)	208±26 (0·04)	1983±121 (N.S.)

Table 3. *Oxidoreduction state and concentration of the nicotinamide nucleotides in rat hepatomas induced by 3′MeDAB*

The nicotinamide nucleotides were extracted and assayed by the method of Greenbaum *et al.* (1965*a*). The results are expressed as means ± S.E.M. *P* values are given in parentheses (see Table 1).

	No. of animals	Body wt. (g.)	Liver wt. (g.)	Concn. (μg./g. wet wt.) NAD+	NADH	$\frac{NAD^+}{NADH}$ ratio	NADP+	NADPH	$\frac{NADPH}{NADP^+}$ ratio
Control	6	718±42	15·7±0·75	422±67	154±13·2	2·69±0·3	36·0±9·3	262·0±45·7	10·15±2·8
Hepatomas	6	772±23	—	127±51 (0·002)	31±7·7 (< 0·001)	3·74±0·89 (N.S.)	12·2±1·6 (< 0·001)	34·1±8·9 (< 0·001)	4·73±1·6 (0·02)

Table 4. *Total cellular concentrations of NAD and NADP and the activities of the NMN adenyltransferase and NAD+ kinase in rat hepatomas induced by 3'MeDAB*

The nicotinamide nucleotides and enzymes were assayed by the methods of Greenbaum *et al.* (1965*a*,*c*). The results are expressed as means ± S.E.M. *P* values are given in parentheses (see Table 1).

	No. of animals	Body wt. (g.)	Liver wt. (g.)	ΣNAD (μg./g.)	NMN adenylyltransferase (μg. of NAD formed/g./hr.)	ΣNADP (μg./g.)	NAD+ kinase (μg. of NADP formed/g./hr.)
Control	6	718±42	15·7±0·75	576±78	3955±191	297·0±46	1963±338
Hepatomas	6	772±23	—	151±52 (<0·001)	1949±171 (<0·001)	44·3±10·4 (<0·001)	963±165 (<0·001)

Table 5. *Oxidoreduction state and concentration of the nicotinamide nucleotides in the livers of rats given ethionine for 2 weeks*

Nucleotides were extracted and assayed as described by Greenbaum *et al.* (1965*a*). The values are given as means ± S.E.M. *P* values are given in parentheses (see Table 1).

	No. of animals	Body wt. (g.)	Liver wt. (g.)	Concn. (μg./g. wet wt.) NAD+	NADH	NAD+/NADH ratio	NADP+	NADPH	NADPH/NADP+ ratio
Control	6	162±7·8	8·40±1·0	313±13	83·9±10·1	4·12±0·71	19·3±0·8	138±16·0	7·63±1·4
Ethionine-treated	6	140±10·3	6·66±0·7	276±19 (N.S.)	65·4±4·8 (0·03)	4·92±0·86 (N.S.)	25·8±4·7 (N.S.)	127±6·5 (N.S.)	5·2±2·1 (N.S.)

Shimoyama, Kori, Usuki, Lan & Gholson (1965) also found that the activity of this enzyme is decreased in hepatomas derived from a 3'MeDAB-induced primary hepatoma.

Hepatomas produced by ethionine

Long-term treatment of rats with ethionine also produces hepatomas. The effects of this agent on the concentrations of nicotinamide nucleotides and on the activities of NMN adenylyltransferase and NAD^+ kinase in the precancerous phase were therefore studied.

Concentration of the nicotinamide–adenine dinucleotides. The concentrations of the oxidized and reduced forms of NAD and NADP in the livers of rats treated with ethionine for 2 weeks are shown in Table 5. At this short time-interval, ethionine had little effect on the concentration of any of the nicotinamide nucleotides; only that of NADH was lower in the ethionine-treated group.

Activities of NMN adenylyltransferase and of NAD^+ kinase. The feeding of ethionine for 2 weeks decreased the activity of NMN adenylyltransferase significantly but had no effect on the activity of NAD^+ kinase (Table 6).

Effects of α-naphthyl isothiocyanate

α-Naphthyl isothiocyanate differs from the other agents used, in that long-term feeding does not produce a hepatoma, but does produce a cell proliferation of the bile duct which is neither destructive nor invasive, in contrast with the hepatomas produced by the other agents used above.

Concentration of the nicotinamide–adenine dinucleotides. The effects of this agent on the nicotinamide nucleotides are shown in Table 1. α-Naphthyl isothiocyanate significantly decreases the amount of NAD^+ but has no effect on the amount of NADH. It increased the $NADP^+$ content by a factor of over 2 but, so great is the variability of response of $NADP^+$ to this agent, the increase was not statistically significant. The NADPH concentration is lowered significantly to about 65% of the control.

Activities of NMN adenylyltransferase and of NAD^+ kinase. The effects of α-naphthyl isothiocyanate on the activities of these two enzymes are shown in Table 2. Neither enzyme is significantly decreased in activity in the livers of rats treated with α-naphthyl isothiocyanate.

Changes in the 'extra, acid-labile' form of $NADP^+$

In view of the findings of Burch *et al.* (1963) on the existence of a form of $NADP^+$ that is acid-labile and not found in the usual type of acid extract used for assay of nicotinamide nucleotides, this 'extra' form was also estimated in the livers of

Table 6. *Total cellular concentrations of NAD and NADP and the potential activities of the NMN adenylyltransferase and NAD^+ kinase in the livers of rats given ethionine for 2 weeks*

The nicotinamide nucleotides and enzymes were assayed by the methods of Greenbaum *et al.* (1965*a*,*c*). The results are expressed as means ± S.E.M. *P* values are given in parentheses (see Table 1).

	No. of animals	Body wt. (g.)	Liver wt. (g.)	∑NAD (μg./g.)	NMN adenylyltransferase (μg. of NAD formed/g./hr.)	∑NADP (μg./g.)	NAD^+ kinase (μg. of NADP formed/g./hr.)
Control	6	162 ± 7·8	8·4 ± 1·0	397 ± 16	3885 ± 375	158 ± 16	2565 ± 330
Ethionine-treated	6	140 ± 10·3	6·66 ± 0·7	342 ± 22 (0·074)	3223 ± 212 (0·05)	153 ± 10 (N.S.)	3087 ± 387 (N.S.)

Table 7. *Concentration of two forms of NADP+ (differing with respect to their stability in 0·1 N-hydrochloric acid) in the livers of rats treated with azo-dyes, α-naphthyl isothiocyanate or ethionine*

The normal NADP+ (*a*) was extracted and assayed by the method of Greenbaum *et al.* (1965*a*). Acid-labile NADP+ (*b*) was extracted by the method of Burch *et al.* (1963), and estimated as above. The results are expressed as means ± S.E.M. *P* values are given in parentheses (see Table 1).

Compound given	No. of animals	Body wt. (g.)	Liver wt. (g.)	NADP+ extracted in 0·1 N-HCl (μg./g.) (*a*)	NADP+ extracted in 0·02 N-H_2SO_4–0·1 M-Na_2SO_4 (μg./g.) (*b*)	Quotient (*b*/*a*)	'Extra' NADP+ (μg./g.) (*b*−*a*)
None	7	297±26	11·9±0·2	20·6±3·8	56·0±6·9	2·72	35·5±5·9
3′MeDAB	8	278±16	12·5±0·73	17·7±3·3 (N.S.)	32·6±5·9 (0·01)	1·84	17·95±6·7 (0·03)
4′FDAB	8	269±13	10·3±1·0	15·0±2·1 (0·04)	33·3±3·7 (0·002)	2·21	16·5±4·5 (0·004)
α-Naphthyl isothiocyanate	6	269±13·6	13·8±1·0	45·7±16·2 (N.S.)	32·3±4·8 (0·004)	0·71	None
None	6	162±8	8·4±1·0	19·3±0·8	40·4±3·2	2·09	20·0±4·0
Ethionine	6	140±10	6·7±0·7	25·8±4·7 (N.S.)	54·7±7·4 (0·056)	2·12	28·9±4·9 (0·02)

Table 8. *Concentrations of two forms of NADP+ (differing with respect to their stability in 0·1 N-hydrochloric acid) in rat hepatomas induced by 3′MeDAB*

(*a*) Normal NADP+; (*b*) acid-labile NADP+ (see Table 7). The results are expressed as means ± S.E.M. *P* values are given in parentheses (see Table 1).

	No. of animals	Body wt. (g.)	Liver wt. (g.)	NADP+ extracted in 0·1 N-HCl (μg./g.) (*a*)	NADP+ extracted in 0·02 N-H_2SO_4–0·1 M-Na_2SO_4 (μg./g.) (*b*)	Quotient (*b*/*a*)	'Extra' NADP (μg./g.) (*b*−*a*)
Controls	6	718±42	15·7±0·75	36·0±9·3	55·8±8·9	1·55	20·1±5·95
Hepatomas	6	722±23	—	12·35±1·6 (0·001)	8·31±2·79 (0·001)	0·67	−2·33±1·57 (0·001)

rats that had been treated for 14–28 days with one of the four agents used above and in hepatomas. The results of the assay of this form of $NADP^+$ are shown in Tables 7 and 8. Also included in Tables 7 and 8 are the results of assays of the normal form of $NADP^+$, which is present in 0·1 N-acid extracts. Short-term treatment with either of the two azo-dyes or with α-naphthyl isothiocyanate significantly decreased the concentration of $NADP^+$ that is extracted in weak acid and also the 'extra' $NADP^+$ (Table 7, columns 6 and 8). α-Naphthyl isothiocyanate was particularly active with respect to the 'extra' $NADP^+$, there being no 'extra' $NADP^+$ present at all in tissues treated with this agent. Ethionine had the reverse effect, in that it increased both the $NADP^+$ extractable in weak acid and the 'extra' $NADP^+$. In hepatomas the $NADP^+$ extracted in weak acid is actually less than that extracted in 0·1 N-hydrochloric acid.

Effect of the concentration of NAD^+ on the synthesis of $NADP$ by NAD^+ kinase

To investigate this effect the assay system for NAD^+ kinase (Greenbaum *et al.* 1965*c*) was used except that the amount of NAD^+ was varied from 0·44 mg. to 13·2 mg. in 1 ml. of reaction mixture. The NADPH formed at each concentration of NAD^+ was extracted and assayed as above. The results for this experiment are shown in Fig. 1.

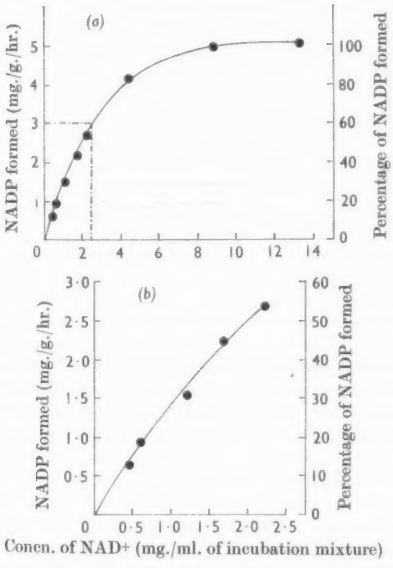

Fig. 1. Effect of NAD^+ concentration on the activity of rat-liver NAD^+ kinase. The liver was homogenized (1:10) in 0·15 M-KCl–0·16 mM-$KHCO_3$ and centrifuged for 135000 g-min. The supernatant was used as the enzyme source as described by Greenbaum *et al.* (1965*c*). Fig. 1(*b*) is an expanded version of the area marked in Fig. 1(*a*).

DISCUSSION

One of the more interesting manifestations of the advent and development of malignancy in hepatic cells is the loss of nicotinamide nucleotides, in particular of NADP. In a fully developed hepatoma the NADP concentration is less than one-fifth of that of a normal liver. The loss of NADP is readily investigated since its synthesis requires only three components, all readily measurable. These are the two substrates, NAD^+ and ATP, and the enzyme NAD^+ kinase. In the present investigation both NAD^+ kinase and NAD^+ have been measured. Table 9 summarizes our findings and shows the concentrations of the nicotinamide nucleotides and the activities of the enzymes concerned in their synthesis, expressed as percentages of the control values. It is apparent that no correlation exists between the measured activity of NAD^+ kinase and the concentration of the nucleotide. 4′FDAB, for instance, causes a loss of over 40% of the NADP in a precancerous liver, but has no effect on the activity of NAD^+ kinase. Similarly, with 3′MeDAB and α-naphthyl isothiocyanate there were falls in NADP concentration with no corresponding change in NAD^+-kinase activity. Although it is true that the profound loss of NADP that occurs in hepatomas is accompanied by a severe decrease in NAD^+-kinase activity, it is not certain that there is a cause-and-effect relationship here. It is probable that by the time a hepatoma has developed widespread secondary changes have occurred in the liver and the loss of NAD^+-kinase activity may merely reflect a generalized depletion of some enzyme activities.

In contrast with the lack of correspondence between enzyme activity and NADP concentration, there appears to be a reasonably close parallelism between the concentrations of NAD and NADP (Table 9, columns 1 and 3). The relationships between these two can be evaluated by reference to Fig. 1, which shows the relationship between the activity of NAD^+ kinase and the amount of NAD present. Since the enzyme is measured as the maximum potential activity with saturating concentration of substrate, it follows that a truer measure of intracellular activity would be the enzyme activity at the NAD concentration actually found in the cell. From Fig. 1 it is possible to express this activity as a percentage of the maximum rate. Thus from the measured NAD content of the cell and the measured maximum potential activity of NAD^+ kinase an estimate can be formed of the actual rate

Table 9. *Concentrations of coenzymes and activities of enzymes expressed as percentages of control values*

The values for the coenzymes and enzymes are calculated from Tables 2, 4 and 6. The values in **bold** type are significantly different from the control value.

Compound given	ΣNAD	NMN adenylyl-transferase	ΣNADP	NAD^+ kinase
14–28 days				
3′MeDAB	**73**	92	**77**	94
4′FDAB	**59**	**77**	**56**	101
Ethionine	**86**	**83**	97	100
α-Naphthyl isothiocyanate	73	89	72	120
Hepatomas				
3′MeDAB	**26**	**49**	**15**	**49**

Table 10. *Comparison between the observed concentrations of NADP in livers of rats treated with 3′MeDAB, 4′FDAB, α-naphthyl isothiocyanate or ethionine, and the concentration calculated from the tissue NAD content*

ΣNAD and the 'observed' ΣNADP are taken from Tables 2, 4 and 6. NAD^+-kinase activities are taken from Tables 2, 4 and 6.

Experimental condition	ΣNAD (μg./g.)	NAD^+ kinase (μg. of NADP/g./hr.) (maximum potential activity)	NAD^+ kinase (μg. of NADP/g./hr.) (corrected to NAD content of tissue = *A*)*	ΣNADP (μg./g.) Observed	ΣNADP (μg./g.) Calc.†
1. Control group	506	1990	318	287	—
3′MeDAB-treated	370	1865	232	221	210
4′FDAB-treated	300	2020	194	161	175
α-Naphthyl isothiocyanate-treated	370	1983	246	208	222
2. Control group	400	3087	402	158	—
Ethionine-treated	340	2565	308	153	121
3. Control group	575	1963	344	300	—
3′MeDAB hepatomas	150	963	46	44	40

* NAD^+-kinase activities corrected to activities at the cellular concentration of NAD are calculated from Fig. 1.

† Calculated from:

$$\frac{A \text{ for experimental group}}{A \text{ for control group}} \times \text{NADP content of control group}$$

of NAD^+-kinase activity. Let this be called *A*. Assuming that the concentration of NADP in a cell is in equilibrium with the rate of its formation by NAD^+ kinase, it is possible to write:

$$\text{NADP content of experimental animal} = \frac{A \text{ for experimental animal}}{A \text{ for control animal}} \times \text{NADP content of control animal}$$

The results of such a calculation are shown in Table 10. There is a sufficient degree of correspondence between the expected concentration of NADP, calculated from the NAD content, and the actual concentration of NADP to suggest that the cellular content of NAD may regulate the formation of NADP. However, it is unlikely that this form of control extends over a wide range. In particular, it is unlikely that the NAD concentration controls the NADP concentration when the NAD concentration is supranormal. This is suggested by the fact that when the cellular NAD concentration is increased to supranormal values by injection of nicotinamide there is only a small increase in the concentration of NADP (Kaplan, Goldin, Humphreys, Ciotti & Stolzenbach, 1956; Wintzerith *et al.* 1961; Gordon & Reichlin, 1962; Greengard, Quinn & Reid, 1964). It is thus possible that at low cellular concentrations of NAD it is this component which is rate-limiting for NADP synthesis, but at high concentrations of NAD it is clearly not the

rate-limiting compound. This suggests that the third component of the NADP-synthesizing system, ATP, may well become so. Evidence in favour of this view can be derived from the work of Dietrich & Yero (1964), who found that the injection of nicotinamide into mice led to a rise in the hepatic NAD concentration but a fall in the hepatic ATP concentration. Thus injection of or feeding with nicotinamide leads to increased amounts of NAD accompanied by lower ATP concentrations, and under these conditions NADP synthesis is not greatly stimulated (Kaplan *et al.* 1956; Wintzerith *et al.* 1961; Gordon & Reichlin, 1962; Greengard, *et al.* 1964), which suggests a competition for the available ATP by the enzymes synthesizing either NAD or NADP. This possibility is discussed further below. There is also evidence from the work of Stekol, Bedrak, Mody, Burnette & Somerville (1963) that the availability of ATP can influence the rate of NAD synthesis after feeding with nicotinamide.

As a result of the above considerations, it seemed important to investigate the possible role of ATP as a regulator of NADP synthesis. To study the effect of a lowered ATP concentration, advantage was taken of the observation of Shull (1962) that injection of ethionine into rats led to a decrease of the hepatic ATP concentration within 2hr. that persisted for up to 6hr. In such ethionine-treated rats it was found that the NAD concentration was only slightly lowered 5hr. after treatment, whereas the NADP concentration was lowered by about 40% and the NAD^+-kinase activity was unchanged (Greenbaum, Clark & McLean, 1964). In this experiment, neither the very small fall in NAD concentration nor the virtually unchanged activity of NAD^+ kinase could account for the decline in the NADP concentration, and the most likely cause was the lowered ATP concentration resulting from the ethionine injection. To explain the differential effects of ATP on the reactions leading to the synthesis of NAD and NADP, both of which are ATP-requiring, Greenbaum *et al.* (1964) considered the different K_m values for these two reactions. For half maximum activity, NAD^+ kinase requires a concentration of ATP that is approx. 150 times as great as is required by NMN adenylyltransferase. Thus, when ATP is limiting, the limitation will press more hardly on NADP synthesis than on NAD synthesis. This difference could account for the failure to find appreciable NADP synthesis in nicotinamide-treated rats and in ethionine-treated rats. It is also noteworthy that after injection of ethionine the concentrations of both NAD and NADP fall together, but that NAD recovers earlier, returning to normal by 5hr. after the injection, whereas NADP remains depressed (J. B. Clark & S. Pinder, unpublished work). The NAD recovery occurs at a time when the concentration of ATP is still below normal.

The precancerous liver, as well as the fully developed hepatoma, both contain subnormal amounts of NAD. All carcinogens decreased the concentration of this coenzyme and the greatest effect was produced by the most potent of the carcinogens, 4′FDAB (Table 9). There is very little relationship between the activity of NMN adenylyltransferase and the concentration of NAD in these treated livers except, perhaps, that 4′FDAB, which produces the greatest fall in NAD concentration, also has the greatest effect on NMN adenyltransferase. This lack of correspondence between enzyme and product is not surprising in view of the results of Shimoyama *et al.* (1965), which show that in hepatomas the probable rate-limiting step is the formation of nicotinic acid ribonucleotide.

The biological significance of the form of NADP that is extractable in weak acid (Burch *et al.* 1963) is obscure, but it may be noted that all the agents, with the exception of ethionine, decreased the concentration of this form in the livers of treated rats. Neubert, Schulz & Hoehne (1964) suggested that this acid-labile NADP is a haem-catalysed reoxidation of NADPH, which is the predominant form of NADP in the cell, and consider that the 'extra', acid-labile, NADP would be related to the $NADPH/NADP^+$ quotient, being high when this quotient is high. In fact, we have found considerable changes in the amount of acid-labile NADP in the precancerous livers of 3′MeDAB- and 4′FDAB-treated rats even though there is no appreciable change in the $NADPH/NADP^+$ quotient. However, for α-naphthyl isothiocyanate and in the hepatomas the suggested relationship is found, i.e. there is a considerable fall in the $NADPH/NADP^+$ quotient and the acid-labile NADP virtually disappears. However, previous studies of the content of oxidized and reduced forms of NADP in the livers of rats subjected to a number of treatments (Greenbaum, Clark & McLean, 1965*b*) also failed to show the relationship suggested by Neubert *et al.* (1964). Further studies on the acid-labile form of NADP are needed before its biological source and role can be assessed.

We thank Dr E. Reid for providing some of the tumour material. We are grateful to the Medical Research Council for a Research Grant to J. B. C. The work has been supported, in part, by a grant to The Middlesex Hospital Medical School from the British Empire Cancer Campaign.

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Effects of Prolonged Incubation of Isolated Fat Cells on their Response to Hormones Stimulating Lipolysis and Glucose Metabolism

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(*Received* 26 *July* 1965)

1. The metabolism of isolated fat cells from parametrial adipose tissue of starved normal rats was studied during 8hr. incubation. 2. There was a three- to eight-fold increase in conversion of glucose into carbon dioxide, fatty acids and glycerol during the fourth to eighth hours of incubation in 4% albumin buffer over that seen during the first 4hr. of incubation. 3. The addition of growth hormone and dexamethasone to fat cells at the start of the incubation period accelerated lipolysis during the first 4hr. of incubation but no further effect was seen during the fourth to eighth hours of incubation. Addition of growth hormone and dexamethasone to fat cells that had been incubated for 4hr. did not accelerate lipolysis during the next 4hr. whether fat cells were incubated with or without glucose. 4. Fat cells incubated for prolonged periods also displayed a reduced sensitivity to the lipolytic action of adrenocorticotrophic hormone. 5. During prolonged incubation there was no damage to the cells as judged by the retention of two soluble cytoplasmic enzymes, lactate dehydrogenase and malate dehydrogenase, within the cells.

Isolated fat cells prepared by incubation of adipose tissue with bacterial collagenase can be separated from other cells, since they float to the top of the incubation medium (Rodbell, 1964). Unlike dispersed cells from the liver, which cease respiration (Laws & Stickland, 1956) and leak many soluble cytoplasmic enzymes such as lactate dehydrogenase and malate dehydrogenase (Takeda, Ichihara, Taniok & Inoue, 1964), adipose cells can metabolize glucose for at least 4hr. and more glucose is metabolized during the second to fourth hours of incubation than during the first 2hr. (Fain, Kovacev & Scow, 1965). In addition, adipose cells respond to hormones that affect adipose tissue (Rodbell, 1965).

The present studies were designed to examine the effect of incubating fat cells for periods up to 8hr. on the metabolism of glucose and their response to hormones.

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MATERIALS AND METHODS

Female Sprague–Dawley rats of the NIH strain (140–160g.) were fed on a high-fat (40%) diet (Simon, Scow & Chernick, 1961) for 5–7 days before the experiments. The rats were starved overnight and killed by decapitation. Isolated fat cells were prepared by a modification of the procedure of Rodbell (1964); the parametrial adipose tissue was incubated for only 1 hr. in albumin–bicarbonate buffer solution, without glucose, containing bacterial collagenase (Worthington Biochemicals Corp., Freehold, N.J., U.S.A.). A 4% solution of bovine fraction V albumin (lot 22, 312; Armour Laboratories, Kankakee, Ill., U.S.A.) was made up fresh daily in bicarbonate buffer and adjusted to pH 7·4 in an atmosphere of $CO_2 + O_2$ (5:95). The bicarbonate buffer contained the following: NaCl (127mM), KCl (2·7mM), $CaCl_2$ (1·4mM), $MgCl_2$ (0·5mM), $NaHCO_3$ (12mM) and NaH_2PO_4 (4mM). The albumin–bicarbonate buffer was filtered through a 0·45μ Millipore filter before use. Fat cells were incubated in 1oz. polyethylene bottles in a metabolic shaker at 37° in an atmosphere of $CO_2 + O_2$ (5:95). In those experiments in which glycerol release was measured the plastic bottles were rinsed before use since many lots of the Nalgene bottles were contaminated with glycerol. Incubations were performed in duplicate or

triplicate in each experiment and zero-time samples were taken after 5 min. equilibration.

All statistical comparisons are based on paired comparisons and the significance of the difference was tested by a non-parametric sign test (Siegel, 1956); this test was used since the distribution of some of the data was not normal.

Assay procedures. Samples of the medium were analysed for glycerol by a fluorimetric modification of the procedure of Vaughan (1962) and for free fatty acids by a modification of the procedure of Dole & Meinertz (1960). In some experiments 0·2 μc of D-[U-^{14}C]glucose (New England Nuclear Corp., Boston, Mass., U.S.A.) was added to each bottle, making a final specific activity of 0·04 μc/μmole. The $^{14}CO_2$ was determined by the procedure of Fain, Scow & Chernick (1963) and labelled glyceride glycerol and fatty acids by the procedures of Rodbell (1964). The amount of cells added per flask was based on their total fatty acid content.

Assays of lactate dehydrogenase and malate dehydrogenase were performed on samples of the incubation medium or homogenates prepared by rupture of cells in a Vortex homogenizer. The assay medium for lactate dehydrogenase contained the following: triethylamine hydrochloride (250 mM), adjusted to pH 7·6 with NaOH, NADH (0·03 mM) and pyruvate (10 mM), in a total volume of 1 ml. The assay medium for malate dehydrogenase contained: glycylhydrazine (200 mM), adjusted to pH 9·5 with NaOH, NAD^+ (0·05 mM) and malate (10 mM), in a total volume of 1 ml. In each assay the substrate was added last. Enzyme activity was based on the initial change in the native fluorescence of the nicotinamide nucleotides, measured with an Aminco Fluoromicrophotometer. The wavelength of the incident light was 340 mμ and the secondary filter transmitted light above 410 mμ.

Materials. The hormones used were: bovine growth hormone (NIH-GH-B3) obtained from the Endocrinology Study Section of the National Institutes of Health: Oxycel-purified adrenocorticotrophic hormone (100 units/mg.) purchased from Wilson Laboratories, Chicago 9, Ill., U.S.A.; soluble insulin, Iletin U-40 (Eli Lilly and Co.) purchased locally; and dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-4-pregnene-3,20-dione), obtained from Merck, Sharp and Dohme Research Laboratories, Rahway, N.J., U.S.A. Protein hormones were diluted in albumin buffer.

RESULTS

The conversion of glucose into carbon dioxide was fourfold greater, into fatty acid eightfold greater and into glyceride glycerol 2·5-fold greater by fat cells during the fourth to eighth hours of incubation than during the first 4 hr. (Table 1). The increase in basal glucose metabolism during the second 4 hr. resembled the effect produced by the addition of insulin during the first 4 hr. (Table 1). When insulin was added in the 4–8 hr. period there was an effect which was less than that seen after insulin addition during the 0–4 hr. period. But the sum of the basal increase in glucose metabolism and the insulin effect from 4 to 8 hr. was only

Table 1. *Effects of incubation time and insulin (1 milliunit/ml.) on the metabolism of* [U-^{14}C]*glucose in isolated fat cells*

Fat cells (5 mg./flask) were incubated for 4 or 8 hr. in 2 ml. of medium with [U-^{14}C]glucose (final concn. 2·8 mM). Results are given as means ± S.E.M. of five experiments and *P* was calculated by the non-parametric sign test (Siegel, 1956).

Time of incubation (hr.)	Insulin + [^{14}C]glucose added at (hr.)	Glucose converted (μmoles/g. of adipose tissue/4 hr.)									
		Carbon dioxide				Fatty acids				Glyceride glycerol			
		Control	Insulin	Insulin effect	*P*	Control	Insulin	Insulin effect	*P*	Control	Insulin	Insulin effect	*P*
4	0	6 ± 2	41 ± 23	35	0·03	0·2 ± 0·04	4·0 ± 1·6	3·8	0·03	12 ± 3	49 ± 20	37	0·03
8	4	26 ± 12	34 ± 17	8	0·03	1·6 ± 0·07	2·6 ± 1·0	1·0	0·03	29 ± 9	35 ± 12	6	0·03
Effect due to preincubation on activity 4 to 8 hr.		+ 20 (*P* = 0·03)				+ 1·4 (*P* = 0·03)				+ 17 (*P* = 0·03)			

slightly less than the maximal response obtained with insulin over 0–4hr. incubation.

The addition of 0·01–1·0μg. of growth hormone/ml. in the presence of 0·016μg. of dexamethasone/ml. has been shown to accelerate lipolysis in fat cells (Fain *et al.* 1965). The lipolytic effect of these hormones is slow in onset since lipolysis was much greater during the second to fourth hours of incubation than during the first 2hr. of incubation (Fain *et al.* 1965). The results in Table 2 indicate that the addition of growth hormone and dexamethasone at the start of the incubation period stimulates fatty acid release during the first 4hr. but no further effect was seen when the incubation period was extended to 8hr. No effect of growth hormone and dexamethasone on either fatty acid or glycerol release could be obtained during the fourth to eighth hours of incubation even when the hormones were added at the beginning of the fourth hour of incubation (Table 3).

To decide whether the increase in basal glucose metabolism was responsible for the failure of action of growth hormone and dexamethasone, the action of these hormones was examined in albumin buffer without glucose. The results are shown in Table 4. The addition of growth hormone and dexamethasone stimulated lipolysis in fat cells incubated without glucose if added at the start of the first 4hr. incubation period, in confirmation of previous studies (Fain *et al.* 1965). However, if growth hormone and dexamethasone were added at the

Table 2. *Effects of growth hormone and dexamethasone on fatty acid release from incubated fat cells*

Fat cells (20mg./flask) were incubated for 4 or 8hr. in 2ml. of medium containing glucose (2·8mM) as described in the text. Concentration of additions at zero time: growth hormone, 1·0μg./ml.; dexamethasone, 0·016μg./ml. Results are given as means ±S.E.M. of eight experiments. *P* represents probability of difference between means of corresponding pairs calculated by the non-parametric sign test.

Time during which fatty acid release was measured (hr.)	Fatty acid release (μmoles/g.) Control	Increase due to hormones	*P*
0–4	66	73±17	0·004
4–8	32	7±28	>0·1

Table 3. *Effects of growth hormone and dexamethasone on lipolysis when added at zero time or after 4hr. incubation with glucose*

Fat cells (20mg./flask) were incubated for 4 or 8hr. in 2ml. of medium containing glucose (2·8mM). Growth hormone (1·0μg./ml.) and dexamethasone (0·016μg./ml.) were added at times indicated in the Table. Results are given as means ±S.E.M. of six experiments and *P* was calculated by the non-parametric sign test.

Time over which fatty acid release was measured (hr.)	Time of addition of hormones (hr.)	Fatty acid release (μmoles/g.) Control	Increase due to hormones	*P*	Glycerol release (μmoles/g.) Control	Increase due to hormones	*P*
0–4	0	67	51±22	<0·01	35	18±7	<0·01
4–8	4	30	−5±8	>0·2	40	1±1	>0·2

Table 4. *Effects of growth hormone and dexamethasone on fatty acid release from incubated cells in the absence of glucose*

Fat cells (4mg./flask) were incubated for 4 or 8hr. in 2ml. of medium without glucose. Growth hormone (1·0μg./ml.) and dexamethasone (0·016μg./ml.) were added at times indicated in the Table. Results are shown as means ±S.E.M. of six experiments and *P* was calculated by the non-parametric sign test.

Time over which fatty acid release was measured (hr.)	Time of addition of hormones (hr.)	Fatty acid release in 4hr. after hormone addition (μmoles/g.) Control	Increase due to hormones	*P*
0–4	0	308	160±80	0·015
0–8	4	101	−34±67	>0·6

Table 5. *Effect of adrenocorticotrophic hormone on fatty acid release by fat cells*

Fat cells (3·5 mg./flask) were incubated in 2 ml. of medium containing glucose (2·8 mM). Adrenocorticotrophic hormone was added 1 hr. before the end of the incubation period and the results are shown as the means of seven experiments. Increases due to the hormone are given as means ± S.E.M. of the paired differences.

	Fatty acid release (μmoles/g.)			
Incubation time (hr.)	4		8	
Hormone (μg./ml.)	Mean	Increase due to hormone	Mean	Increase due to hormone
0	135		425	
0·002	370	+235±85	335	−90±65
0·01	840	+705±175	615	+190±140
0·1	1400	+1265±200	1175	+750±120

Table 6. *Release of lactate dehydrogenase and malate dehydrogenase from isolated fat cells (5 mg./flask) into incubation medium*

Enzyme activity is expressed as μmoles of NADH oxidized or NAD^+ reduced/min./ml. of medium or homogenate. Results are given as means ± S.E.M. Experimental details are given in the text.

		Lactate dehydrogenase			Malate dehydrogenase		
Duration of incubation (hr.)	No. of experiments	Activity in medium	Activity in cell homogenate	Release (%)	Activity in medium	Activity in cell homogenate	Release (%)
0	4	0·1±0·04	1·2±0·05	9	0·2±0·01	3·6±0·05	5
4	6	0·1±0·01	1·2±0·02	8	0·3±0·04	3·1±0·1	10
8	7	0·1±0·01	1·0±0·2	10	0·2±0·01	3·0±0·1	9

start of the second 4 hr. of incubation they had no effect on lipolysis.

The effect of prolonged incubation on the sensitivity of fat cells to another lipolytic hormone, adrenocorticotrophic hormone, was examined. Table 5 records the response of isolated fat cells to three concentrations of adrenocorticotrophic hormone added at the third and seventh hours of incubation. In cells that had been incubated for 7 hr., as opposed to those that had been incubated for 3 hr., there was no lipolytic effect of 0·001 or 0·01 μg. of adrenocorticotrophic hormone/ml. If the concentration of the hormone was increased to 0·1 μg./ml. a lipolytic effect was apparent in cells incubated for 7 hr. before its addition.

The possibility that there was leakage of soluble enzymes out of the fat cell as has been described for isolated liver cells (Takeda *et al.* 1964) was examined. The presence of two soluble cytoplasmic enzymes, lactate dehydrogenase and malate dehydrogenase, was measured in the medium and cells. After 8 hr. incubation there was no fall in the lactate-dehydrogenase content of the cells and less than a 20% decrease in malate dehydrogenase. No appreciable increases in amounts of these enzymes were detected in the incubation medium over the 8 hr. period of incubation (Table 6).

DISCUSSION

The evidence from this study indicates that there is a marked increase in glucose metabolism by isolated fat cells during the fourth to eighth hours of incubation and a reduced sensitivity to lipolytic hormones. Similar effects on glucose metabolism can be obtained by the addition of insulin at the start of a 4 hr. incubation period. Fain, Kovacev & Scow (1966) have noted that in the presence of insulin there is no lipolytic effect of growth hormone and dexamethasone whether cells are incubated with or without glucose and that the effect of small but not of large concentrations of adrenocorticotrophic hormone is blocked by insulin. The similarity in the effects of prolonged incubation and of insulin suggests that insulin might be responsible for the rise in glucose metabolism during prolonged incubation. Alp & Recant (1964) found that preparations of human plasma albumin stimulated glucose metabolism by adipose tissue during a 2 hr. incubation period. However, if

there is insulin in the albumin it is difficult to understand why the effect of insulin is not manifest during the first 4hr. of incubation.

Herrera, Phillips & Renold (1965) have noted that even after incubation of adipose tissue from starved rats for longer periods than those used in the present experiments there was no marked increase in glucose metabolism. But there was an increase in glucose metabolism with time if the tissue was incubated with pyruvate, insulin and glucose, or serum. In the present experiments the increase in glucose metabolism of adipose cells on prolonged incubation occurred in the absence of added serum, insulin, or pyruvate.

The increase in glucose metabolism during prolonged incubation could conceivably be due to spontaneous changes in the plasma membrane of the fat cells which allow increased entry of glucose and also decrease net lipolysis. If a membrane change of this sort occurs the evidence suggests it is not due to non-specific damage during incubation as has been found for incubated liver cells (Takeda *et al.* 1964), since there is no leakage of soluble cytoplasmic enzymes from the cells into the incubation medium.

The authors are grateful to Dr R. O. Scow, Dr M. Rodbell and Dr S. S. Chernick for their interest and encouragement. D. J. G. is indebted to the Mental Health Research Fund, London, for a Travelling Fellowship.

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Biochem. J. (1966) **98**, 562

Characterization of Ribonucleases and Ribonuclease Inhibitor in Subcellular Fractions from Rat Adrenals

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(*Received* 9 *August* 1965)

1. The presence of two RNA-degrading enzymes, one with optimum activity at pH 5·6 (acid ribonuclease) and the other with optimum activity at pH 7·8 (alkaline ribonuclease), in rat adrenals has been demonstrated. The acid ribonuclease was localized in the mitochondrial fraction whereas the alkaline ribonuclease was present in mitochondria as well as in the supernatant fraction. Freezing and thawing of mitochondria and treatment with Triton X-100 gave a three- to four-fold increase in acid-ribonuclease activity, whereas the mitochondrial alkaline-ribonuclease activity was practically unaffected. 2. The amount of free ribonuclease in the adrenal supernatant was small. Treatment of the supernatant fraction with *N*-ethylmaleimide resulted in release of large amounts of ribonuclease activity, indicating the presence of a ribonuclease inhibitor having reactive thiol groups. 3. Considerable amounts of free ribonuclease inhibitor in excess over the bound alkaline ribonuclease are present in the rat-adrenal supernatant fraction. The inhibitor is heat-labile and non-diffusible. A 400–500-fold purification of the ribonuclease inhibitor was achieved by ammonium sulphate fractionation, treatment with calcium phosphate gel and DEAE-cellulose chromatography. It is concluded that the adrenal inhibitor is protein in nature, similar to the inhibitor present in rat liver.

Roth (1956*a*, 1957) has reported the presence of an inhibitor for ribonuclease activity in rat-liver supernatant fraction. Shortman (1961) has demonstrated from purification studies of the rat-liver inhibitor that the inhibitor activity is associated with a fraction that is mostly protein. Eichel, Figueroa & Goldenberg (1961) have provided evidence for the presence of ribonuclease inhibitor in rat adipose tissue.

There have been a number of attempts to assess the possible role of the ribonuclease inhibitor in RNA metabolism and protein synthesis by determining the changes in the concentrations of ribonuclease and ribonuclease inhibitor after altered physiological states in the organism. Thus the changes in inhibitor activity have been studied in rat liver after whole-body X-irradiation (Roth, 1956*b*) or during regeneration after partial hepatectomy (Shortman, 1962*b*) and in different cancerous tissues (Brody, 1957; Roth, 1962). Girija, Pradhan & Sreenivasan (1965) have reported on the correlation observed between altered protein-synthetic activity and the ribonuclease inhibitor concentrations in livers of rats maintained on protein-free diet for 3 weeks. Smeaton, Elliott & Coleman (1965) have found a powerful inhibitor of ribonuclease activity in the soluble fraction of disrupted cell preparations of a strain of *Bacillus subtilis*. They have also provided preliminary evidence for the protein nature of this bacterial ribonuclease inhibitor.

Thus these observations call for a detailed understanding of the cellular inhibitors of ribonuclease from a wide variety of sources, with special reference to their intracellular location, specificity and mode of inhibition. The present work reports on the distribution of ribonuclease activity in the subcellular fractions of rat adrenals and the presence of an inhibitor of ribonuclease activity in the adrenal supernatant fraction. The inhibitor has been purified by chromatography on DEAE-cellulose columns.

EXPERIMENTAL

Animals. Livers and adrenals were obtained from male Wistar rats, maintained on the laboratory stock diet, and weighing 250–300g.

Preparation of subcellular fractions. The tissues were dissected and homogenized with 10 vol. of 0·25 M-sucrose, previously chilled, in a Potter–Elvehjem glass homogenizer. The isolation of subcellular fractions was done according to the procedure of Schneider & Hogeboom (1945). The

homogenate was centrifuged at 500*g* in an International model PR–2 refrigerated centrifuge to remove nuclei and unbroken cells. Mitochondria were separated at 5000*g* for 15min. These were washed twice with 10vol. of 0·25M-sucrose and recentrifuged under the same conditions. The resulting mitochondrial pellet was suspended in 10vol. of 0·25M-sucrose for the assay of free ribonuclease activity. Total ribonuclease activity was determined after freezing and thawing a 1:10 suspension of mitochondria in water or after treatment with Triton X-100 (0·1%, v/v) at 4° for 20min.

The supernatant obtained after sedimenting mitochondria was spun in a Spinco model L ultracentrifuge at 105000*g* for 1hr. and the resulting supernatant fraction was used in the studies on the ribonuclease inhibitor.

Determination of ribonuclease activity. Ribonuclease activity was determined as described by Brody (1957), by following the release of acid-soluble nucleotides on incubation of 2·5mg. of dialysed yeast RNA with a 1:10 homogenate or equivalent cell fraction in a final volume of 3·0ml. Incubations at 37° were carried out in 20mM-tris–HCl buffer, pH7·8, for the determination of alkaline-ribonuclease activity and in 20mM-acetate buffer, pH5·6, for the assay of acid-ribonuclease activity. For the supernatant enzyme, preincubation of the supernatant fraction with *N*-ethylmaleimide (final concn. 3mM) was carried out for 30min. before incubation with the substrate. The reaction was stopped by the addition of 3·0ml. of 10% (w/v) trichloroacetic acid containing uranyl acetate (0·5%), and the acid-soluble nucleotides were determined by measuring the extinction at 290mμ in a Beckman model DU spectrophotometer, trichloroacetic acid giving negligible extinction at this wavelength. The amount of enzyme releasing acid-soluble nucleotides equivalent to 1 extinction unit under the above conditions is expressed as 1 ribonuclease unit.

Assay of ribonuclease inhibitor. The system for the assay of ribonuclease inhibitor (Roth & Wojner, 1961) consisted of 0·2ml. of supernatant fraction, 0·15ml. of 50mM-EDTA, pH7·8, 0·125μg. of crystalline pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), 1·0ml. of veronal–acetate buffer (50mM with respect to both sodium diethylbarbiturate and sodium acetate), pH7·8, and 1·0ml. of 1% RNA solution in a final volume of 3·0ml. Incubation was carried out for 30min. at 37°. The reaction was stopped by the addition of 3·0ml. of precipitating reagent [N-HCl in 76% (v/v) ethanol containing $LaCl_3,7H_2O$ (0·5%)]. Release of acid-soluble nucleotides was estimated by the change in extinction at 260mμ. One unit of inhibitor is equivalent to the concentration causing 50% inhibition of 0·005μg. of crystalline pancreatic ribonuclease.

Purification of ribonuclease inhibitor. Sheep adrenals contained inhibitor activity comparable with that from rat adrenals. The inhibitor from sheep adrenals resembled the rat-liver inhibitor in all its characteristics, being heat-labile, non-diffusible and containing active thiol groups.

A 30g. portion of sheep adrenals was homogenized in 3vol. of 0·25M-sucrose and the supernatant fraction obtained by spinning the homogenate at 105000*g* was treated with solid $(NH_4)_2SO_4$ to give 35% saturation. The precipitate was removed by centrifugation at 60000*g* in the Spinco model L ultracentrifuge for 20min. Then $(NH_4)_2SO_4$ was added to the supernatant to give 55% saturation and the mixture was centrifuged at 60000*g* for 20min. The pellet was dissolved in 20ml. of cold water and this was treated with calcium phosphate gel, prepared according to the method of Swingle & Tiselius (1951). The inhibitor was eluted from the gel with 20–25ml. of 0·2M-phosphate buffer, pH6·4. The eluate was diluted to give a phosphate concentration of 10mM and NaCl and EDTA were added to give concentrations of 0·15M and 1mM respectively. This solution was applied to DEAE-cellulose columns. The chromatography on DEAE-cellulose columns was essentially as described by Shortman (1961). The column was washed with 50ml. of buffered saline [NaCl (0·15M), potassium phosphate buffer, pH6·4 (10mM), and EDTA (1mM)]. The elution was carried out with a linear gradient of 0·15–1·5M-NaCl in 10mM-phosphate buffer, pH6·4, containing EDTA (1mM). The eluate was collected in 5·0ml. fractions.

Determination of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS AND DISCUSSION

Two apparently distinct enzymes from various tissues that hydrolyse RNA have been described by Roth (1954), Stevens & Reid (1956), Maver & Greco (1956) and Zytko, De Lamirande, Cantero & Allard (1958). The activities of these have been differentiated on the basis of pH optima and stability to heat and acid, one enzyme being termed

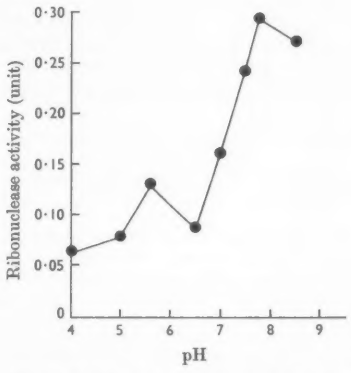

Fig. 1. Effect of pH on the ribonuclease activity of rat-adrenal homogenate. Samples of rat-adrenal homogenate equivalent to 50mg. of tissue were incubated with 2·5mg. of dialysed yeast RNA at 37° for 30min. in veronal–acetate buffers in the pH range 4·0–8·5. The reaction was stopped by the addition of 3·0ml. of 10% (w/v) trichloroacetic acid containing uranyl acetate (0·5%), and the acid-soluble nucleotides were determined by measuring the extinction at 290mμ. One unit of ribonuclease activity is the amount of enzyme releasing acid-soluble nucleotides equivalent to 1 extinction unit under the above conditions. The plot represents the average of three different experiments.

alkaline ribonuclease (optimum pH 7·8–8·2, heat-stable and acid-stable) and the other acid ribonuclease (optimum pH 5·2–5·8, heat-labile and acid-labile). Fig. 1 illustrates the pH–activity curve of ribonuclease in adrenal homogenate. The curve reveals two peaks of activity, one at pH 5·6 and another at pH 7·8. These results indicate that rat adrenals contain both acid and alkaline ribonucleases with pH optima similar to those reported for the ribonucleases of rat liver (Roth, 1957).

The distribution of acid and alkaline ribonucleases in the subcellular fractions of rat liver and adrenals is shown in Table 1. The acid-ribonuclease activity is localized in the mitochondrial fraction, the activity in the supernatant fraction being negligible. Alkaline-ribonuclease activity is present in mitochondria as well as in the supernatant fraction. The ribonuclease activity in the supernatant fraction is low. But, on the addition of *N*-ethylmaleimide, a 3–4-fold increase in the activity is observed. The same extent of release of supernatant enzyme was obtained when *p*-chloromercuribenzoate was present in the incubation system at a final concentration of 0·4 mM. These results are similar to those reported for rat liver (Roth, 1958). Calculation of the distribution of alkaline-ribonuclease activity between mitochondria and supernatant shows that about 60% of the ribonuclease activity could be recovered in the supernatant fraction, after taking into account the ribonuclease activity released on treatment with *N*-ethylmaleimide, and about 20% in the mitochondria. Experiments showed that mitochondrial activity is not increased on treatment of mitochondria with *N*-ethylmaleimide. However, other treatments, such as freezing and thawing and treatment with detergents like Triton X-100, increase the mitochondrial ribonuclease activity. The increase is mainly in the acid-ribonuclease activity, alkaline-ribonuclease activity being unaffected by these treatments (Table 1).

These results demonstrate that, as in rat liver, a major part of the ribonuclease activity in the adrenals is present in a latent form, the supernatant enzyme occurring in combination with an inhibitor, which could be inactivated by thiol-binding reagents, and the mitochondrial acid-ribonuclease activity present presumably as a membrane component.

The significance of the intracellular location of acid and alkaline ribonucleases of mitochondria is not known, though attempts have been made to study the characteristics of the two enzymes (Stevens & Reid, 1956; Zytko *et al.* 1958; Maver & Greco, 1962). Nodes, Reid & Whitcut (1962), working with purified enzymes, found minor differences in their specificities towards synthetic substrates. However, one difference between acid and alkaline ribonucleases is that, whereas acid ribonuclease is not inhibited by the rat-liver inhibitor, all the three mammalian alkaline ribonucleases tested, namely those of rat-liver mitochondria and supernatant as well as the pancreatic enzyme, are all inhibited (Shortman, 1962*a*).

Although mitochondrial alkaline-ribonuclease activity is present in a free form whereas the supernatant alkaline-ribonuclease activity is present along with the inhibitor presumably in combination, as inferable from observations with use of thiol-binding agents, it has been shown that purified alkaline ribonucleases of mitochondria and super-

Table 1. *Distribution of ribonuclease activity in rat-adrenal fractions and comparison with liver cell fractions*

Free ribonuclease was determined by following the release of acid-soluble nucleotides on incubation of a 1:10 homogenate or equivalent cell fraction with 2·5 mg. of dialysed yeast RNA. Total ribonuclease activity of mitochondria at pH 5·6 or 7·8 was determined in the same manner after freezing and thawing a 1:10 suspension in water (*) or after treatment with 0·1% (v/v) Triton X-100 (†). The supernatant activity was assayed with and without preincubation with *N*-ethylmaleimide (final concn. 3 mM). The activities are expressed as ribonuclease activity units/50 mg. of tissue or equivalent cell fraction. One unit of ribonuclease activity is the amount of enzyme releasing acid-soluble nucleotides equivalent to 1 extinction unit under the above conditions. The results are averages of four independent determinations.

	Ribonuclease activity (unit)						
	Homogenate	Mitochondria				Supernatant	
		Free activity		Total activity			
Tissue	Free activity pH 7·8	pH 5·6	pH 7·8	pH 5·6	pH 7·8	−*N*-Ethylmaleimide pH 7·8	+*N*-Ethylmaleimide pH 7·8
Adrenals	0·305	0·100	0·108	0·398* 0·412†	0·108* 0·112†	0·095	0·280
Liver	0·250	0·124	0·124	0·342* 0·400†	0·118* 0·121†	0·080	0·220

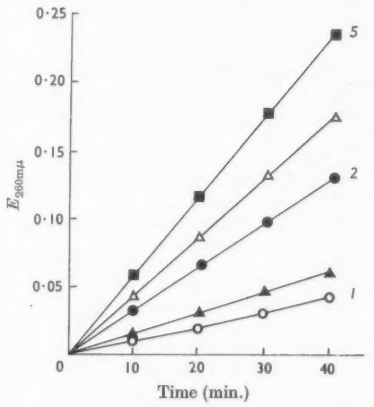

Fig. 2. Effect of *N*-ethylmaleimide on the inhibition of crystalline pancreatic ribonuclease by adrenal supernatant fraction. The assay system consisted of 10·0ml. of 50mM-veronal–acetate buffer, pH7·8, 10ml. of 1% dialysed yeast RNA, 1·0μg. of crystalline pancreatic ribonuclease, 4·0ml. of 1:10 supernatant and, where indicated, *N*-ethylmaleimide (final concn. 3mM). The total volume was 25ml. Samples (4·0ml.) were added after 0, 10, 20, 30 and 40min. to 4·0ml. of precipitating reagent [N-HCl in 76% (v/v) ethanol containing $LaCl_3,7H_2O$ (0·5%)]. The supernatant was diluted 1:50 and readings were taken at 260mμ. The plots represent the averages of three independent experiments. Curve *1*, activity of adrenal supernatant alone; curve *2*, activity of supernatant fraction preincubated with *N*-ethylmaleimide; curve *3*, activity of crystalline pancreatic ribonuclease; curve *4*, activity of crystalline pancreatic ribonuclease in the presence of supernatant; curve *5*, activity of crystalline pancreatic ribonuclease in the presence of supernatant and *N*-ethylmaleimide.

natant are identical in their specificity towards substrates (Nodes *et al.* 1962; Beard & Razzell, 1964).

A time-course study of the action of *N*-ethylmaleimide on the adrenal supernatant fraction is shown in Fig. 2. The ribonuclease activity in the supernatant fraction is low (curve *1*). On the addition of *N*-ethylmaleimide, a threefold increase in activity is observable (curve *2*), indicating a bound form of ribonuclease in the supernatant fraction, and that this is released on inactivation of the inhibitor by thiol-binding reagents like *N*-ethylmaleimide. However, the supernatant fraction is not saturated with respect to ribonuclease, since considerable amounts of crystalline enzyme may be inactivated by the addition of supernatant fraction (curves *3* and *4*). This inhibition is overcome in the presence of *N*-ethylmaleimide (curve *5*).

The results of two typical experiments to test the heat-sensitivity of the inhibitor are given in Table 2. The inhibitor is relatively heat-sensitive, being completely destroyed after heating for 5min. at 65°. Together with the fact that the inhibitor is

Table 2. *Effect of heating the supernatant fraction on the activity of ribonuclease inhibitor*

Samples (2ml.) of adrenal supernatant fraction were placed in test tubes and heated in a water bath for 5min. at the different temperatures. After heating, the samples were cooled and assayed for inhibitor activity. The system for the assay of ribonuclease inhibitor consisted of 0·2ml. of supernatant fraction, 0·15ml. of 50mM-EDTA, pH7·8, 0·125μg. of crystalline pancreatic ribonuclease, 1·0ml. of 50mM-veronal–acetate buffer, pH7·8, and 1·0ml. of 1% RNA solution in a final volume of 3·0ml. The reaction was stopped after 30min. incubation at 37° by adding 3·0ml. of precipitating reagent [N-HCl in 76% (v/v) ethanol containing $LaCl_3,7H_2O$ (0·5%)]. Release of acid-soluble nucleotides was estimated by the change in extinction at 260mμ. The unit of inhibitor is equivalent to the inhibitor concentration causing 50% inhibition of 0·005μg. of crystalline pancreatic ribonuclease. The results are averages of two experiments.

Temperature	Inhibitor activity (units/mg. of protein)	Decrease of inhibitor activity (%)
37°	13·6	—
45	12·4	9
55	5·8	57
65	0	100

Table 3. *Purification of sheep-adrenal ribonuclease inhibitor*

The supernatant fraction was subjected to $(NH_4)_2SO_4$ precipitation and treatment with calcium phosphate gel according to the procedure described in the text. The eluate from calcium phosphate gel was adsorbed on DEAE-cellulose columns. The conditions of adsorption and elution are given in the text. One unit of inhibitor is equivalent to the inhibitor concentration causing 50% inhibition of 0·005μg. of crystalline pancreatic ribonuclease. The results are averages of two independent experiments carried out with separate batches of sheep adrenals.

Stage	Activity (units/ml.)	Specific activity (units/mg. of protein)	Recovery (%)
Supernatant	420	15	100
55% satd. $(NH_4)_2SO_4$ precipitate	420	52	85
Eluate from calcium phosphate gel	630	174	58
Eluate from DEAE-cellulose column chromatography	5640	6300	80

non-diffusible in crude as well as in purified preparations, the observation suggests that it may be protein in nature.

The inhibitor activity could be purified several hundred-fold by employing methods used for the purification of proteins. An analysis of the results of a standard purification procedure is presented in Table 3. Initial purification was carried out by combined ammonium sulphate precipitation and calcium phosphate gel treatment. The specific activity of the inhibitor preparation thereby increased 10–12-fold; the partially purified inhibitor was next passed through a column of DEAE-cellulose. All of the inhibitor activity was retained on the column. Elution was carried out with a linear gradient of 0·15–1·5M-sodium chloride buffered at pH 6·4 with tris. The highest inhibitor concentrations were obtained in fractions 13, 14, 15 and 16. These eluate fractions also contained most of the 280mμ-absorbing material. A 400–500-fold purification was thus attained.

Though an analysis of the purified inhibitor preparation has not been undertaken, previous studies also indicate that protein forms the major constituent in purified rat-liver inhibitor preparations. No other component was present to any considerable extent (Shortman, 1962*a*).

The protein nature of the inhibitor (Shortman, 1961), as well as its lability *in vivo* (Roth, 1956*b*; Shortman, 1962*b*; Girija *et al.* 1965), lead one to speculate that the inhibitor may play an important role in the regulation of RNA metabolism, especially in view of recent reports implicating ribonuclease as the enzyme involved in the degradation of rapidly turned over RNA in bacterial (Artman & Engelberg, 1964) as well as in animal systems (Harris, 1963).

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The Enzymic Oxidation of Chlorogenic Acid and some Reactions of the Quinone Produced

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(*Received* 23 *August* 1965)

1. Partially purified preparations of tobacco-leaf *o*-diphenol oxidase (*o*-quinol–oxygen oxidoreductase; EC 1.10.3.1) oxidize chlorogenic acid to brown products, absorbing, on average, 1·6 atoms of oxygen/mol. oxidized, and evolving a little carbon dioxide. 2. The effect of benzenesulphinic acid on the oxidation suggests that the first stage is the formation of a quinone; the solution does not go brown, oxygen uptake is restricted to 1 atom/mol. oxidized, and a compound is produced whose composition corresponds to that of a sulphone of the quinone derived from chlorogenic acid. 3. Several other compounds that react with quinones affect the oxidation of chlorogenic acid. The colour of the products formed and the oxygen absorbed in their formation suggest that the quinone formed in the oxidation reacts with these compounds in the same way as do simpler quinones. 4. Some compounds that are often used to prevent the oxidation of polyphenols were tested to see if they act by inhibiting *o*-diphenol oxidase, by reacting with quinone intermediates, or both. 5. Ascorbate inhibits the enzyme and also reduces the quinone. 6. Potassium ethyl xanthate, diethyldithiocarbamate and cysteine inhibit the enzyme to different extents, and also react with the quinone. The nature of the reaction depends on the relative concentrations of inhibitor and chlorogenic acid. Excess of inhibitor prevents the solution from turning brown and restricts oxygen uptake to 1 atom/mol. of chlorogenic acid oxidized; smaller amounts do not prevent browning and slightly increase oxygen uptake. 7. 2-Mercaptobenzothiazole inhibits the enzyme, and also probably reacts with the quinone; inhibited enzyme is reactivated as if the inhibitor is removed as traces of quinone are produced. 8. Thioglycollate and polyvinylpyrrolidone inhibit the enzyme. Thioglycollate probably reduces the quinone to a small extent.

The intermediates formed when polyphenols* are oxidatively polymerized inactivate or bind some of the more labile enzymes and plant viruses. In particular, cucumber mosaic virus is inactivated in extracts of infected tobacco leaves during the oxidation of chlorogenic acid, and getting infective extracts requires extraction methods that prevent this oxidation (Harrison & Pierpoint, 1963; Pierpoint & Harrison, 1963). It is not known which of the oxidation products is the inactivator and which parts of the virus are sensitive. The first steps in answering these questions require knowledge of the mechanism by which chlorogenic acid is oxidized, and of the reactions that are likely to occur between intermediates in the process and components of leaf extracts.

If the oxidation follows a course similar to the oxidation of catechol by mushroom enzymes (Mallette, 1950), the initial step would be the formation of a quinone with the uptake of 1 mol. of oxygen/mol. of quinone formed. Non-enzymic reactions could then convert half of the quinone into hydroxyquinone and regenerate chlorogenic acid from the rest, and polymerization of the hydroxyquinone would produce the brown end products:

$$\text{Chlorogenic acid} + [\text{O}] \xrightarrow{\text{Enzyme}} \text{quinone} \quad (1)$$

$$\text{Quinone} + \text{H}_2\text{O} \rightarrow \text{hydroxychlorogenic acid} \quad (2)$$

$$\text{Quinone} + \text{hydroxychlorogenic acid} \rightarrow \text{hydroxyquinone} + \text{chlorogenic acid} \quad (3)$$

$$\text{Chlorogenic acid} + [\text{O}] \xrightarrow{\text{Enzyme}} \text{quinone} \quad (1a)$$

$$\text{Hydroxyquinone} \rightarrow \text{polymer} \quad (4)$$

* In the usual nomenclature of these phenols, the prefixes poly- and di- refer to the number of hydroxyl groups attached to the benzene ring. *o*-Quinol, as used in the systematic name for polyphenol oxidase recommended by the I.U.B. Commission on Enzymes, refers to any substance containing the *o*-dihydroxybenzene group.

The present paper supplements the incomplete evidence of Clayton (1959) for the formation of a quinone during the oxidation of chlorogenic acid by tobacco-leaf *o*-diphenol oxidase (*o*-quinol–oxygen oxidoreductase; EC 1.10.3.1). It presents evidence that this quinone reacts with a range of compounds containing nitrogen and sulphur much as do simpler quinones, and that these reactions may involve additional oxygen absorption. In addition, a number of substances that are commonly used to prevent the oxidation of polyphenols are shown to affect this process either by inhibiting the enzyme, by combining with the quinone intermediate, or both.

EXPERIMENTAL

o-Diphenol oxidase preparations. Leaves of *Nicotiana tabacum* (var. Xanthi-nc) had their mid-veins removed and were stored, generally overnight but occasionally for a few days, at −15°. Batches (20g.) of frozen material were disrupted in 200ml. of cold (−15°) acetone by treatment in an MSE Ato-Mix blender for 2min., and the protein precipitate was collected by filtration, washed with more cold acetone and dried over P_2O_5 in a vacuum desiccator. The acetone-dried powder was extracted for 30min. at 0° with 35 times its own weight of 0·1M-tris buffer, pH7·2, containing cysteine (0·05–0·1M); the residue, separated by centrifugation, was re-extracted with a quarter of the initial volume of buffered cysteine. The two extracts were combined and mixed with approx. 2·5 times their volume of cold (0–5°) saturated $(NH_4)_2SO_4$ solution. The precipitated protein was usually left overnight, then collected by centrifugation, dissolved in approx. 20ml. of 0·2M-tris buffer, pH7·0, and dialysed overnight against two 4l. lots of water. It was clarified by centrifugation, if necessary, before being stored at −15°.

This procedure, which is based on the first stages of the method of purification described by Clayton (1959), was carried out in the cold room (0–5°) as far as possible. It usually produced 20–30ml. of solution containing about 1mg. of protein N/ml. Sometimes this solution was slightly brown, indicating that, in spite of the precautions taken, there was some oxidation of leaf polyphenols during the preparation.

Manometric estimations. The action of *o*-diphenol oxidase was followed at 30° by conventional manometry under conditions that measure either initial enzyme activity, or the total amount of oxygen absorbed/mol. of substrate oxidized. Substances could therefore be tested as inhibitors of *o*-diphenol oxidase, and also for reaction with the quinone intermediates formed during the oxidation.

When enzyme activity was measured, each manometer flask generally contained enzyme preparation (0–90μg. of protein N), tris buffer, pH7 (200μmoles), chlorogenic acid (24μmoles) and water to 3ml.; changes, as when the pH was altered or tris buffer was replaced by phosphate buffer, are mentioned in the text. Carbon dioxide was absorbed by KOH in the centre wells, and the flasks were shaken at about 80 oscillations/min. In these conditions the enzyme was almost saturated with substrate; the Michaelis constant is about 2·7mM (Mrs P. Wrench, unpublished work). Oxygen uptake was approximately linear for 10min. after mixing enzyme and substrate. It was measured as a routine for 4 or 5min. when, over the range of protein concentrations used, it was proportional to the amount of enzyme preparation added. Substances tested as inhibitors were added, in solution at pH7, to the main compartments of the flasks. They were therefore incubated with enzyme during the 10min. equilibration period before substrate was tipped in from the side arms.

In the 5min. reaction period the oxygen uptake seldom exceeded 57μl., corresponding to the oxidation of 20% of the added chlorogenic acid to quinone. The bulk of the oxygen is therefore likely to be absorbed in this reaction rather than the oxidation of chlorogenic acid regenerated from the quinone by reactions (2) and (3). To confirm that this was so, most estimations were repeated in the presence of 33mM-benzenesulphinic acid. As shown below, this substance removes quinone from the reaction medium, so preventing its participation in further reactions. Enzyme activities determined in its presence were only slightly lower (5–7%) than those determined without it. Objections to the direct manometric determination of *o*-diphenol oxidase have been raised by Nelson & Dawson (1944); however, the 'chronometric' method of estimation, recommended by Nelson & Dawson (1944), is inappropriate for the tobacco-leaf oxidase, which is inhibited by ascorbate (Fig. 4).

When the total oxygen absorbed/mol. of substrate oxidized was being measured, each manometer flask contained 6μmoles of chlorogenic acid, which was oxidized to completion by an excess (0·2–2·0mg. of protein N) of enzyme preparation. The reaction was followed for 45–60min., but had usually stopped after 20min., when little or no chlorogenic acid could be detected chromatographically in the reaction medium. The enzyme present was at least twice, and more usually 4 times, that necessary to complete the oxidation in 20min. Such an excess made it possible to measure the total oxygen absorbed when chlorogenic acid was completely oxidized in the presence of enzyme inhibitors.

Examination of reaction products. Spectrophotometric examination of reaction media was carried out with either a Unicam SP.500 or an Optica SF4 recording spectrophotometer.

Chromatographic examination was done either on the reaction mixture itself or on ether extracts of it. When the mixture was used the reaction was carried out in dilute buffer or, after careful pH adjustment of the reactants, without buffer. The final solution was then spotted directly on to paper. Ether extracts were made by extracting the reaction medium twice, first after acidifying it to pH4 with N-HCl and again after adjusting the pH to 2. The two extracts were pooled and concentrated before application to paper. Chromatography was performed on sheets (approx. 23cm. × 57cm.) of Whatman 3MM filter paper, with as solvent the upper phase formed from butan-1-ol–acetic acid–water (4:1:5, by vol.). Irrigation was downwards and was continued for 10–22hr., depending on the separation required. After drying, the chromatograms were examined in daylight and in u.v. light. This was repeated after they had been exposed to NH_3 vapour. They were then either sprayed with or dipped into one of the following reagents. (1) $FeCl_3,6H_2O$ (1%, w/v) plus $K_3Fe(CN)_6$ (1%, w/v), which becomes blue with polyphenols and other reducing agents (Barton, Evans & Gardner, 1952). (2) Hoepfner's reagent

[$NaNO_2$ (5%, w/v) in 0·9N-acetic acid followed by N-NaOH], which gives a pink or yellow–orange colour with chlorogenic acid and related polyphenols (Roberts & Wood, 1951). (3) I_2 (0·1N) dissolved in KI (0·12N) to which is added NaN_3 (3%, w/v) immediately before use, this reagent being rapidly bleached by compounds containing C=S or SH groups and, less readily, by disulphides (Feigl, 1954; Chargaff, Levine & Green, 1948). However, results obtained with it were interpreted cautiously as chlorogenic acid and some of its derivatives also decolorize it fairly quickly; presumably I_2 reacts with the unsaturated linkage in their side chains. (4) 2,6-Dichloroquinonechloroimide (0·2%, w/v) in ethanol, which gives yellow–brown colours with some thiols and slowly produces a range of colours with other compounds (Smith & Birchenough, 1958). (5) Hydrated ammonium molybdate (2·5%, w/v) in 0·2N-HCl, which gives purple–blue colours with xanthate (Lederer & Lederer, 1957), but was also found to produce dirty yellow colours with chlorogenic acid and its derivatives. (6) Ninhydrin (0·2%, w/v) in acetone, followed by heating for a few minutes at 80°.

The sulphone formed from benzenesulphinic acid was separated from the reaction medium by a method similar to that used by Pugh & Raper (1927) to isolate *o*-dihydroxydiphenyl sulphone. The reaction medium from five manometer flasks was acidified to pH 4 and extracted with five 20ml. lots of ether. This was repeated after further acidification to pH 2·5. The pooled extracts were evaporated to dryness and the residue was treated with 40ml. of boiling benzene to dissolve any benzenesulphinic acid. The benzene treatment was repeated three times and the final white residue recrystallized from water and dried over P_2O_5. The product was analysed for C, H and S by Weiler and Strauss (Microanalytical Laboratory, Oxford). Its absorption spectrum (Fig. 2) was not altered when it was chromatographed in butanol–acetic acid–water and eluted from the paper with water.

Reagents. Tris buffer was a 0·2M solution of tris adjusted to pH 7 with 2N-HCl. Phosphate buffer was similarly a 0·2M solution of NaH_2PO_4 adjusted to the required pH by conc. HCl or KOH. The chlorogenic acid was a commercial sample from Koch–Light Laboratories Ltd., Colnbrook, Bucks. It contained a minor component that fluoresced blue–grey in u.v. light and had R_F 0·2–0·3 in butanol–acetic acid–water. The contaminant was unaffected by tobacco-leaf extracts and is unlikely to have affected any of the reactions studied.

RESULTS

Oxidation of chlorogenic acid

The oxidation of chlorogenic acid to brown end products by excess of enzyme involved the absorption of 1·4–1·9 atoms of oxygen/mol. of chlorogenic acid oxidized. A little carbon dioxide (less than 0·15mol./mol.) was evolved, especially towards the end of the reaction.

Oxygen uptake during the oxidation of catechol by mushroom *o*-diphenol oxidase can be resolved into two stages at low pH values (Dawson & Nelson, 1938*b*). The first stage is the rapid enzymic oxidation of catechol to *o*-quinone; the second depends on the non-enzymic conversion of quinone into 4-hydroxyquinone, which is slow in acid conditions. The oxidation of chlorogenic acid by tobacco-leaf enzyme followed a slightly different course at pH 4 than at pH 7 (Fig. 1), but gave no unequivocal evidence that the oxidation occurred in two stages.

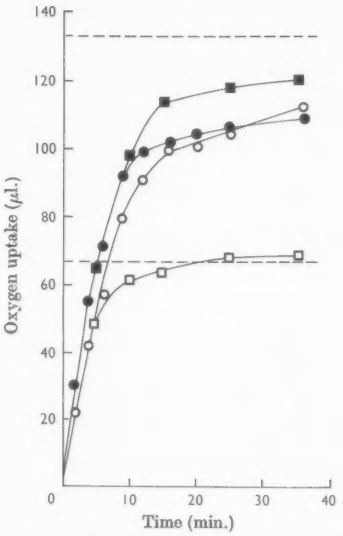


Fig. 1. Effect of pH and of benzenesulphinic acid on the oxidation of chlorogenic acid. In one experiment, chlorogenic acid (6μmoles) was oxidized by enzyme preparation (2mg. of protein N) in 125mM-phosphate buffer at pH 7·6 (●) or pH 4·5 (○). In the other experiment the oxidation (0·74mg. of protein N) occurred in tris buffer at pH 7, with (□) or without (■) 4mM-benzenesulphinic acid. The broken lines show oxygen uptake corresponding to 1 and 2 atoms/mol. of chlorogenic acid oxidized.

The effect of benzenesulphinic acid on the oxidation gave evidence for the formation of quinones. This compound, which has previously been used in studies on the oxidation of monophenols (Pugh & Raper, 1927), reacts with quinones to produce sulphones (Hinsberg & Himmelschein, 1896) by the reaction:

$$C_6H_5 \cdot SO_2H + C_6H_4(O)_2 \rightarrow C_6H_5 \cdot SO_2 \cdot C_6H_3(OH)_2$$

When 3·3–33mM-benzenesulphinic acid was present during the oxidation of 3mM-chlorogenic acid by excess of enzyme, it restricted oxygen uptake to 1 atom/mol. of chlorogenic acid (Fig. 1) and prevented browning. Smaller amounts of benzenesulphinic acid were progressively less effective, but still appeared to combine with the first products of the reaction (Table 1). Benzenesulphinic acid had

Table 1. *Effect of benzenesulphinic acid on the oxidation of chlorogenic acid by tobacco-leaf o-diphenol oxidase*

The oxidation of 9 μmoles of chlorogenic acid was taken to completion by an excess of enzyme preparation (0·3 mg. of protein N in Expt. 1; 0·2 mg. of protein N in Expt. 2) in the presence of different amounts of benzenesulphinic acid.

Expt. no.	Concn. of chlorogenic acid (mM)	Concn. of benzenesulphinic acid (mM)	Oxygen uptake (atoms/mol. of chlorogenic acid oxidized)	Final colour of reaction mixture
1	3	0	1·5	Brown
	0	33·0	—	Colourless
	3	33·0	1·08	Yellow
	3	13·2	0·98	Yellow
	3	3·3	1·08	Yellow
	3	1·0	1·45	Orange–brown
2	3	0	1·59	Brown
	3	1·65	1·12	Pale brown
	3	0·99	1·33	Brown
	3	0·66	1·34	Brown
	3	0·33	1·50	Brown

little or no effect on *o*-diphenol oxidase as judged by its effect on the initial rate of oxidation. A small (5%) inhibition, occasionally observed with 33 mM-benzenesulphinic acid, can be explained in terms of a prevention of the secondary stages of the oxidation (reactions 2, 3 and 1a) which occur to a small extent in these estimations of initial reaction rate.

The compound formed between benzenesulphinic acid and the oxidation product of chlorogenic acid is yellow. After acidification it was readily extracted into ether, and on chromatography it migrated as a single spot (R_F about 0·74) with a characteristic yellow–green fluorescence in u.v. light. Exposure to ammonia intensified its yellow colour and also its fluorescence. Hoepfner's reagent turned it orange–yellow. These properties are similar to those of chlorogenic acid, which, however, migrated more slowly (R_F 0·57); they are unlike those of benzenesulphinic acid, which moved at a similar speed. It resembled both these compounds in becoming blue with the ferric chloride–ferricyanide spray, but differed in that it gave a blue colour also with ferric chloride. This compound was not formed when either chlorogenic acid or benzenesulphinic acid was omitted from the reaction medium, and only in trace amounts in the absence of enzyme.

By making use of its solubility in ether and insolubility in hot benzene, this compound was extracted from the reaction mixture and freed from most of its contaminants. A sample, recrystallized once from water, had the composition C, 51·7; H, 4·6; S, 6·2%; loss in weight on drying at 98°, 2·8%. The sulphone formed from benzenesulphinic acid and the *o*-quinone related to chlorogenic acid

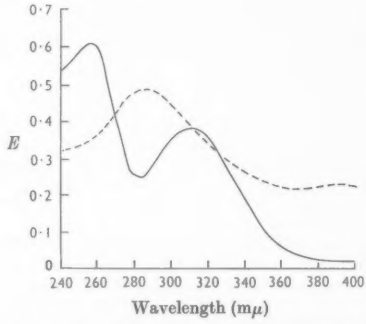


Fig. 2. Spectrum of the sulphone formed from benzenesulphinic acid during the oxidation of chlorogenic acid. The recrystallized material was dissolved (14·6 μg./ml.) in water (——) and N-NaOH was added to a final concentration of 2·5 mN (– – – –).

($C_{22}H_{22}O_{11}S$; see Scheme 1) requires C, 53·5; H, 4·5; S, 6·5%; its monohydrate requires C, 51·6; H, 4·7; S, 6·3%; loss in weight on drying, 3·4%. The ultraviolet spectrum of this compound in water (Fig. 2) has peaks at 254 and 309 mμ. Assuming that it is the monohydrated sulphone, the values of ϵ at these wavelengths are about 21 000 and 13 400 respectively. The addition of alkali displaces both these peaks to longer wavelengths (Fig. 2) in a manner that depends on the concentration of alkali and which alters with time.

Toluenesulphinic acid affected the oxidation of chlorogenic acid in the same way as did benzene-

sulphinic acid. The sulphone formed moved a little slower on chromatography than did the sulphone of benzenesulphinic acid.

Effect of substances that react with quinones on the oxidation of chlorogenic acid

Many substances are known to react with quinones (Mason, 1955) and the effect of some of these on the oxidation of chlorogenic acid was studied.

Aniline. Aniline has previously been used to trap quinones formed during the oxidation of phenol by mushroom *o*-diphenol oxidase (Pugh & Raper, 1927). It inhibited the initial rate of tobacco-leaf *o*-diphenol oxidase by 20% at a concentration of 33mM. However, at a similar concentration, and in the presence of an excess of enzyme, it increased the oxygen absorbed by small amounts of chlorogenic acid to approx. 3atoms/mol. Red compounds appeared almost as soon as the reagents were mixed, and the final colour was a deep cherry-red. Larger amounts of aniline had the same effect except that the final products were dark brown and tended to be precipitated from solution. Aniline thus reacts with the quinone of chlorogenic acid as it does with simpler quinones (Pugh & Raper, 1927). It is likely that at least two molecules of aniline are substituted through their nitrogen atoms into the aromatic nucleus of the quinone (Scheme 1), and in the presence of more concentrated aniline the quinone groups of the dianilidoquinone combine with more aniline to give dark coloured anils.

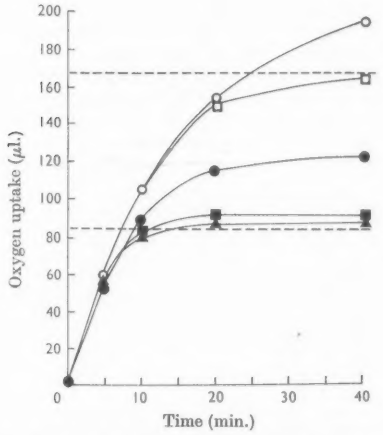


Fig. 3. Effect of nitrogen-containing substances on the oxidation of chlorogenic acid. Chlorogenic acid (7·5μmoles) was oxidized at pH8 by excess of enzyme preparation (400μg. of protein N) as described in the text (●). The nitrogen-containing substances (90μmoles), tested either singly or in combination, were: DL-proline (□); glycine (○); sodium barbiturate (▲); sodium barbiturate plus DL-proline (■); sodium barbiturate plus glycine (■). The broken lines at 84 and 168μl. show oxygen uptake corresponding to 1 and 2 atoms/mol. of chlorogenic acid oxidized.

Glycine. When glycine (30mM) was present during the oxidation of small amounts (3mM) of chlorogenic acid by an excess of enzyme, it affected both oxygen uptake and colour development (see also Hess, 1958). Red-brown colours developed immediately the reaction started and these were finally more intense than those obtained without glycine. The absorption of oxygen was prolonged beyond 30min., and the amount absorbed increased to well over 2atoms/mol. of substrate. Both effects were greater at pH8, and it is at this pH that the experiments described in Fig. 3 were conducted. The pigments formed from chlorogenic acid and glycine thus appear to resemble those found during the oxidation of catechol (James, Roberts, Beevers & de Kock, 1948; Jackson & Kendal, 1949). They probably contain one molecule of glycine linked to the aromatic nucleus by its nitrogen atom (Scheme 1), and the oxygen absorbed in excess of that required for their formation (2atoms/mol.) is probably due to their catalysing the oxidative deamination of additional glycine.

Amino acids with secondary amine groups. Proline and sarcosine (30mM) also stimulated oxygen uptake, but the final amount absorbed was approx. 2atoms/mol. of chlorogenic acid (Fig. 3). Deep-purple colours formed immediately the reagents were mixed. The coloured complexes (see Scheme 1) resembled those formed from these amino acids during the oxidation of catechol (Jackson & Kendal, 1949) in a number of respects. Thus they were reversibly decolorized by ascorbic acid, and irreversibly by hydrochloric acid. Hydrogen ions were liberated during their formation in unbuffered solution, and they were formed more rapidly when chlorogenic acid was oxidized at pH7 than when it was oxidized, at almost the same rate, at pH5.

Other amines. Two tertiary amines, triethanolamine and 2-dimethylaminoethanol (30mM), had no effect on the oxidation of chlorogenic acid, judged either by colour formation or oxygen uptake. A substitutable hydrogen on the amine nitrogen atom appears to be necessary for reaction with the enzymically produced quinone, just as Beevers & James (1948) found it necessary for reaction with the quinone produced during catechol oxidation. But it is evidently not the only requirement; several purines and pyrimidines with primary amine groups also did not affect the oxidation.

Heterocyclic nitrogen compounds. *o*-Quinones react readily with indoles that are unsubstituted in the 3-position, to give a series of highly coloured indol-3′-ylquinones (Bu'Lock & Harley-Mason, 1951). The presence of indole (10–20mM) during the oxidation of chlorogenic acid increased oxygen uptake by a small and rather variable amount (10–40%) provided that the pH was below 8. However, the final reaction mixture was brown, and there was no apparent formation of blue indolylquinones. Steric effects are known to limit the formation of these compounds (Bu'Lock & Harley-Mason, 1951), and they may prevent reaction between indole and the quinone produced from chlorogenic acid. Indoles substituted in the 3-position react with quinones to give a different type of product (Bu'Lock & Harley-Mason, 1951); Leopold & Plummer (1961) described the formation of red pigments when indol-3-ylacetic acid and chlorogenic acid were incubated with *o*-diphenol oxidase. In the present experiments also, a purple–red substance with a broad absorption peak between 520 and 530mμ was formed when the oxidation occurred at pH 5·5. The oxygen uptake corresponded to about 4 atoms of oxygen/mol. of chlorogenic acid, suggesting that more than one indole molecule was substituted into the quinone ring. Much less oxygen, about 2 atoms/mol. of chlorogenic acid, was absorbed when the reaction occurred at pH 7, and red compounds were not apparent until the reaction mixture was acidified.

Barbituric acid (30mM) affects chlorogenic acid oxidation just as benzenesulphinic acid does. It completely prevented the formation of brown end products and restricted oxygen uptake to 1 atom/mol. of chlorogenic acid (Fig. 3). In its presence, as in the presence of benzenesulphinic acid (13mM), the characteristic pigment and oxygen-absorption effects of glycine and proline were completely prevented (Fig. 3). Barbiturates, such as sodium barbitone and sodium phenobarbitone, in which the reactive methylene group is substituted, had no effect. It seems likely therefore that it is via this methylene group that one molecule of barbituric acid combines with one molecule of enzymically generated quinone.

Effect of inhibitors on the oxidation of chlorogenic acid

Several substances commonly used to prevent oxidation of polyphenols were tested to see whether they affect the oxidation of chlorogenic acid by inhibiting tobacco-leaf enzyme, by combining with quinone intermediates, or both.

Effects on the enzyme were measured under conditions in which the bulk of the oxygen uptake was from the enzymic conversion of added chlorogenic acid into quinone. Most were repeated in the presence of benzenesulphinic acid (33mM) to ensure that all the oxygen is absorbed in this reaction. Substances were tested for reaction with quinone by adding them to small amounts of chlorogenic acid, which were being completely oxidized by an excess of enzyme, and observing changes in oxygen uptake and browning of the solution. These changes are largest when the tested substances are present in quantities at least equimolar with chlorogenic acid (1–2mM). Inhibitors of *o*-diphenol oxidase can therefore be tested for reaction with quinones provided that at this concentration they do not inhibit the enzyme by more than about 80%. Of the substances tested, only 2-mercaptobenzothiazole was so inhibitory that it could not be tested in this way.

Ascorbate. This compound reduces quinones to phenolic compounds, and has been used in *o*-diphenol oxidase estimations to keep the quinone concentration low (Nelson & Dawson, 1944). However, it inhibits potato *o*-diphenol oxidase (Baruah & Swain, 1953).

Ascorbate also greatly decreased the initial rate at which tobacco-leaf *o*-diphenol oxidase oxidized chlorogenic acid; in the presence of benzenesulphinic acid, a concentration of 2mM halved the rate (Fig. 4). When small amounts of chlorogenic acid were oxidized by an excess of enzyme in the presence of partially inhibiting quantities of ascorbate, the total amount of oxygen absorbed was increased, and the appearance of brown end products was delayed. Both extra oxygen uptake (Table 2) and the delay in browning were proportional to the ascorbate added. The quinone derived

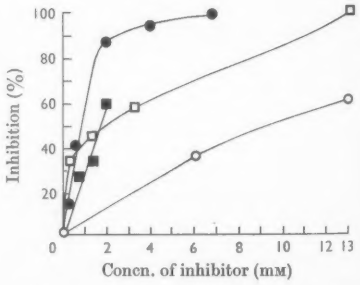


Fig. 4. Inhibition of tobacco-leaf *o*-diphenol oxidase. The effect of thioglycollate (●), ascorbate (□), cysteine (○) and polyvinylpyrrolidone (■) on the initial rate at which the enzyme preparation oxidized chlorogenic acid was determined as described in the Experimental section. Benzenesulphinic acid (33mM) was present in the reaction mixtures except in the experiment with polyvinylpyrrolidone (Kollidon 25; average mol.wt. 24500).

Table 2. *Increase in oxygen uptake when ascorbate is present during the oxidation of chlorogenic acid*

Chlorogenic acid (6 μmoles in Expt. 1; 3 μmoles in Expt. 2) was oxidized to completion by tobacco-leaf *o*-diphenol oxidase (0·88 mg. of protein N in Expt. 1; 0·46 mg. of protein N in Expt. 2) in the presence of different amounts of ascorbate or ascorbate plus benzenesulphinic acid. Neither of these substances gave appreciable oxygen uptake in the absence of chlorogenic acid.

Expt. no.	Benzenesulphinic acid added (μmoles)	Ascorbate added (μmoles)	Oxygen absorbed (μg.atoms)	Oxygen absorbed due to ascorbate (μg.atoms)
1		0	8·3	—
		2	10·8	2·5
		8	16·5	8·2
		12	22·5	14·2
		20	27·3	19·0
2	0	0	5·1	—
	40	0	3·0	—
	0	8	12·6	7·5
	40	8	10·0	7·0

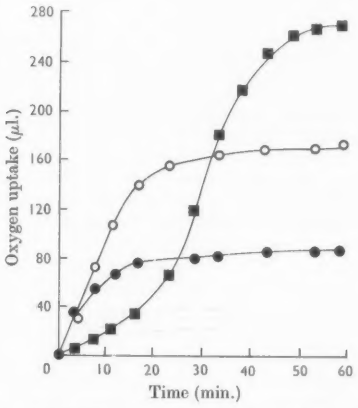


Fig. 5. Reactivation of *o*-diphenol oxidase partially inhibited by ascorbate. Chlorogenic acid (6 μmoles) was oxidized by tobacco-leaf extract (96 μg. of protein N) in the absence (●) and in the presence of 8 μmoles (○) and 20 μmoles (■) of ascorbate.

from chlorogenic acid is therefore, like other quinones, rapidly reduced by ascorbate (Scheme 1); it only accumulates and condenses to brown products when all ascorbate has been oxidized. The increased oxygen uptake caused by 8 μmoles of ascorbate was only slightly diminished by 40 μmoles of benzenesulphinic acid (Table 2), suggesting that the quinone reacts more readily with ascorbate than with benzenesulphinic acid. However, a larger (30-fold) excess of benzenesulphinic acid apparently prevents quinones removing ascorbate, for dilute solutions of ascorbate (1 mM) inhibit *o*-diphenol oxidase more in the presence of benzenesulphinic acid than in its absence. Removal of ascorbate by reaction with quinone probably explains why Farkas, Király & Solymosy (1960) failed to observe an inhibition of tobacco *o*-diphenol oxidase by ascorbate.

Another consequence of the quinone–ascorbate reaction is that *o*-diphenol oxidase, partially inhibited by ascorbate, was, in some conditions, reactivated during the oxidation of chlorogenic acid (Fig. 5). Reactivation of *o*-diphenol oxidases, partially inhibited by a number of other compounds, has previously been observed, but not always attributed to quinone–inhibitor reactions (see Wojtczak & Chmurzynska, 1960).

Potassium ethyl xanthate. This compound chelates copper and other metals and has been used to inhibit copper-containing oxidases (James, 1953; Hill & Mann, 1962). There was a small gas uptake when it was incubated with enzyme preparation. It was equivalent to 20 μl. of oxygen absorbed/hr./80 μmoles of xanthate, but was considerably more in crude leaf extracts. It seems not to be catalysed either by an enzyme or a metal, and it was corrected for in the following experiments. Potassium ethyl xanthate decreased the initial rate of oxidation of chlorogenic acid (Fig. 6), but concentrations less than 5 mM had proportionally more effect than larger ones. This does not depend on the quinone produced during the oxidation; when the oxidation occurred in benzenesulphinic acid, small amounts of xanthate were, proportionally, even more inhibitory (Fig. 6), probably because the benzenesulphinic acid prevented their removal by the reaction with quinone described below.

The complete oxidation of small amounts of chlorogenic acid (3mM) was affected by slightly larger amounts of potassium ethyl xanthate, much as it was by benzenesulphinic acid. The formation of brown pigments was prevented, and oxygen uptake was restricted to about 1 atom/mol. of chlorogenic acid (Fig. 7). This suggests that xanthate combines with the quinones formed during the oxidation (Scheme 1). However, xanthate also had another effect on the oxidation: amounts too small to prevent the formation of brown pigments increased oxygen uptake by 10–20 μl. (Fig. 7). This increase was roughly

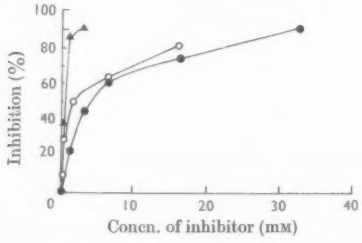


Fig. 6. Inhibition of tobacco-leaf *o*-diphenol oxidase by potassium ethyl xanthate and diethyldithiocarbamate. The rate at which chlorogenic acid (24 μmoles) was oxidized was measured over a 5min. period in the presence of potassium ethyl xanthate (●), potassium ethyl xanthate plus 33mM-benzenesulphinic acid (○), or diethyldithiocarbamate (▲).

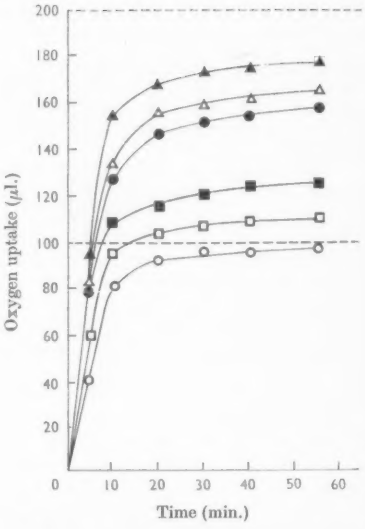


Fig. 7. Effect of potassium ethyl xanthate on the oxidation of chlorogenic acid by tobacco-leaf *o*-diphenol oxidase. Chlorogenic acid (3mM) was oxidized to completion by an excess of enzyme preparation (about 1 mg. of protein N) in the presence of potassium ethyl xanthate of the following concentrations; 0 (●); 0·33mM (△); 1·6mM (▲); 3·3mM (■); 6·6mM (□); 11·5mM (○); 33mM (□). The broken lines at 100 and 200 μl. show the oxygen uptake corresponding to the absorption of 1 and 2 atoms/mol. of chlorogenic acid oxidized.

Table 3. *Amount of potassium ethyl xanthate necessary to inhibit the oxidation of different amounts of chlorogenic acid*

Different amounts of chlorogenic acid were oxidized to completion by an excess of enzyme preparation (0·75mg. of protein N) in the presence of different amounts of xanthate.

Concn. of chlorogenic acid (mM)	Concn. of potassium ethyl xanthate (mM)	Oxygen absorbed (atoms/mol. of chlorogenic acid oxidized)	Colour of final reaction medium
1·0	0	1·95	Brown
1·0	0·6	1·60	Pale brown
1·0	1·0	1·30	Yellow
1·0	3·3	1·30	Yellow
2·0	0	1·50	Brown
2·0	1·0	1·65	Brown
2·0	1·3	1·60	Brown
2·0	2·0	1·20	Yellow
2·0	4·0	1·20	Yellow
3·0	0	1·50	Brown
3·0	2·0	1·56	Brown
3·0	2·5	1·20	Pale brown
3·0	3·0	1·14	Yellow
3·0	4·0	1·10	Yellow

proportional to the amount of xanthate present and it approximated to that expected if the SH groups of xanthate reduced the quinone to chlorogenic acid as does ascorbate. This reduction may also occur to a limited extent with larger amounts of xanthate; oxygen uptake in the presence of 33mM-xanthate was frequently (Fig. 7) a little more than 1 atom/mol. of chlorogenic acid.

The way in which the amount of xanthate necessary to prevent browning and to restrict oxygen uptake depended on the amount of chlorogenic acid oxidized (Table 3) is consistent with the idea that xanthate combines with quinone formed during the oxidation. As 1mM-, 2mM- and 3mM-xanthate respectively prevent browning of 1mM-, 2mM- and 3mM-chlorogenic acid solutions, it also seems that these reactants combine in a 1:1 ratio and have a high affinity for each other. Additional evidence of this affinity is that the increased oxygen absorption, and the colours formed when glycine, proline, sarcosine or aniline (30mM) is present during the oxidation of chlorogenic acid, are prevented by 13·3mM-xanthate.

Two products of the reaction between xanthate and the quinone were detected by chromatographing the reaction mixtures; no trace of either was seen if enzyme, xanthate or chlorogenic acid was omitted. Only one of these products could be extracted by ether from acidified solution, as could the sulphone derived from benzenesulphinic acid. This substance ran faster than chlorogenic acid as a diffuse spot with R_F about 0·8. It appeared as a dark indistinct shadow when viewed under u.v. light, but was a bright yellow in both visible and u.v. light after the chromatograms had been exposed to ammonia vapour. The other compound was detected when the reaction mixture was spotted directly on to chromatogram paper. In u.v. light it was a dull-yellow spot with R_F between 0·15 and 0·25. It too became bright yellow when exposed to ammonia vapour. Both compounds resembled chlorogenic acid in giving blue colours with the ferric chloride–ferricyanide reagent, dirty yellow colours with acid ammonium molybdate and yellow–brown colours with Hoepfner's reagent. The faster-moving one also resembled chlorogenic acid in that it gave a slowly developing (30min.) brown spot when sprayed with 2,6-dichloroquinone-chloroimide and did not bleach the iodine–azide reagent instantaneously. The slower-moving compound, on the other hand, reacted much more quickly with both these sprays, which suggests that it contains free SH or C=S groups.

Diethyldithiocarbamate. Diethyldithiocarbamate is a chelating agent that, although it chelates copper less specifically than does potassium ethyl xanthate (Hill & Mann, 1962), has often been used to inhibit copper-containing enzymes (James, 1953). It decreased the initial rate at which tobacco-leaf *o*-diphenol oxidase oxidized chlorogenic acid more than did potassium ethyl xanthate (Fig. 6). Although inhibition by 1mM-diethyldithiocarbamate was about 80% complete, it was possible, with large amounts of enzyme, to test this substance on complete oxidation of mM-chlorogenic acid. It affected this oxidation just as xanthate did (Table 4). Amounts (1·3–2mM) somewhat greater than those of chlorogenic acid restricted oxygen uptake to about 1 atom/mol. of chlorogenic acid, and kept the mixture yellow or very pale brown. Smaller amounts, which did not prevent browning, increased oxygen uptake beyond the control. This increase was usually 10–20μl., and sometimes more than would be expected from a reduction of the enzymically generated quinone by diethyldithiocarbamate.

A yellow material could be separated chromatographically from the reaction medium after the oxidation of chlorogenic acid in equimolar diethyldithiocarbamate. It had R_F between 0·4 and

Table 4. *Effect of diethyldithiocarbamate on the oxidation of chlorogenic acid by tobacco-leaf* o*-diphenol oxidase*

Chlorogenic acid was oxidized to completion by an excess of enzyme preparation (about 1mg. of protein N) in the presence of different amounts of diethyldithiocarboamate.

Concn. of diethyldithiocarbamate (mM)	Concn. of chlorogenic acid (mM)	Oxygen absorbed (μl.)	Oxygen absorbed (atoms/mol. of chlorogenic acid oxidized)	Colour of reaction mixture
0·0	1	63	1·88	Brown
2·0	1	33	1·00	Yellow
1·3	1	37	1·10	Yellow
1·0	1	53	1·60	Yellow
0·7	1	80	2·40	Brown
2·0	0	2	—	—

0·5, and appeared as a dark absorbing spot in u.v. light. Like chlorogenic acid its colour was intensified by ammonia vapour, and it gave a blue spot with the ferric chloride–ferricyanide reagent. It reacted only weakly with Hoepfner's reagent and very slowly if at all with the iodine–azide spray. It was not formed when chlorogenic acid, diethyldithiocarbamate or enzyme was omitted from the reaction mixture, and was less obvious when higher concentrations of diethyldithiocarbamate were used.

Cysteine. Cysteine has been frequently used as an inhibitor of polyphenol oxidations (e.g. Clayton, 1959). It combines through its sulphur atom with a number of quinones (Mason, 1955), and with intermediates in the oxidation of chlorogenic acid by apple extracts (Henze, 1956; Hulme, 1958; Walker, 1964). It was appreciably oxidized when incubated alone with tobacco-leaf *o*-diphenol oxidase, and oxygen absorptions measured in the experiments described below have been corrected for this.

Cysteine decreased the initial rate at which chlorogenic acid was oxidized by tobacco-leaf extracts (Fig. 4), although it was less effective than the other inhibitors tested. Its effects on the complete oxidation of small amounts (6 μmoles) of chlorogenic acid resemble those of potassium ethyl xanthate and diethyldithiocarbamate, and depend on the ratio of cysteine to chlorogenic acid. When this ratio is more than 1·5, oxygen uptake is restricted to 0·7–0·8 atom/mol. of chlorogenic acid, and the solution does not turn brown (Table 5). In these conditions cysteine probably condenses with the quinone via its SH group (Scheme 1); the oxygen uptake is probably 1 atom of oxygen/mol. of chlorogenic acid, but appears less because the correction for autoxidation of cysteine is over-estimated. When the ratio of cysteine to chlorogenic acid is less than 1·5, browning is not prevented but oxygen uptake is increased (Table 5). The reaction in these conditions may be a reduction of the quinone by the SH groups; but it may also be a reaction of the type between quinone and glycine (Scheme 1). This suggestion receives some support from the fact that cystine similarly increased oxygen consumption without affecting browning of the solution (Table 5).

Chromatographic analysis of solutions in which chlorogenic acid was oxidized in the presence of a fourfold excess of cysteine showed that the disappearance of chlorogenic acid was accompanied by the formation of a slow-moving material (R_F 0·18–0·25) that fluoresced whitish-yellow in u.v. light. It was yellow in both visible and u.v. light after exposure to ammonia fumes, and it gave a blue colour with the ferric chloride–ferricyanide reagent. As it also reacted with ninhydrin and the iodine–azide reagents, it almost certainly contains chlorogenic acid and cysteine groupings. It is probably the same compound as that formed when apple extracts oxidize chlorogenic acid and cysteine (Henze, 1956; Walker, 1964). However, in addition to this substance, small amounts of two other fluorescent compounds were formed, and at least one of them (R_F about 0·6) contained amino nitrogen and, probably, sulphur. There was also ninhydrin-positive material (R_F about 0·15) that

Table 5. *Effect of cysteine and cystine on the enzymic oxidation of chlorogenic acid*

Chlorogenic acid was oxidized to completion by enzyme preparation (57 μg. of protein N) in the presence of cysteine, cystine, or, as a control, benzenesulphinic acid. In the first experiment corrections were made for the autoxidation of cysteine; these certainly over-estimate the oxidation of cysteine that occurs in the presence of chlorogenic acid, and the values marked with an asterisk (*) are therefore low.

Concn. of chlorogenic acid (mM)	Concn. of benzenesulphinic acid (mM)	Concn. of cysteine (mM)	Concn. of cystine (mM)	Oxygen absorbed (μl.)	Oxygen absorbed (atoms/mol. of chlorogenic acid oxidized)	Final colour of reaction mixture
2	0	0·0	—	93	1·40	Brown
2	0	9·0	—	47*	0·70	Yellow
2	0	3·0	—	102*	1·50	Pale brown
2	0	1·8	—	110*	1·65	Brown
2	0	0·6	—	104	1·55	Brown
2	33	0·0	—	66	1·00	Yellow
2	0	—	0·0	93	1·40	Brown
2	0	—	13·0	106	1·60	Brown
2	0	—	6·5	107	1·60	Brown
2	0	—	1·3	104	1·55	Brown
2	0	—	0·6	102	1·50	Brown
2	13	—	0·0	63	0·95	Yellow

was formed non-enzymically from cysteine and chlorogenic acid.

Thioglycollate. Thioglycollate inhibited the initial rate of oxidation of chlorogenic acid more than did xanthate (Fig. 4). Concentrations that did not completely inhibit the enzyme neither prevented the formation of brown pigments during the oxidation of small amounts of chlorogenic acid nor restricted oxygen absorption. After allowing for the small autoxidation of thioglycollate oxygen uptake was, in fact, increased by 10–30%. With less than 1·3mM-thioglycollate, this increase depended roughly on the thioglycollate concentration, and approximated to that expected if thioglycollate, like ascorbate, reduced the enzymically generated quinone back to chlorogenic acid. There was no indication that thioglycollate combines with the enzymically produced quinone as it does with simpler quinones (Mason, 1955). Walker (1964) also failed to find evidence for this reaction with the *o*-diphenol oxidase of apples.

2 - *Mercaptobenzothiazole.* 2 - Mercaptobenzothiazole has been observed by J. K. Palmer & J. B. Roberts (personal communication) to be a potent inhibitor of banana and mushroom *o*-diphenol oxidases, whose action at low concentrations is, however, to delay the onset of oxidation. A similar effect is observed on the oxidation of chlorogenic acid by tobacco-leaf *o*-diphenol oxidase (Fig. 8). Concentrations less than 0·16mM delayed oxidation, which, however, once started, proceeded at approximately the same rate and to approximately the same extent as it did in the absence of inhibitor. The length of the delay was roughly proportional to the amount of inhibitor added, and it was extended indefinitely (more than 22-fold) by 33mM-benzenesulphinic acid. The delay was about twice as long at pH 6·8 as at pH 8·2 (Fig. 8).

J. K. Palmer (personal communication) explains the recovery of inhibited banana *o*-diphenol oxidase as the oxidation of 2-mercaptobenzothiazole to the non-inhibitory disulphide by quinone produced as the oxidation proceeds slowly. It also seems likely that traces of quinone reverse the inhibition of the tobacco-leaf enzyme; the period of inhibition is prolonged by acid conditions, in which the rate of autoxidation of chlorogenic acid is low, as well as by benzenesulphinic acid, which combines with quinones. But the small amounts of mercaptobenzothiazole involved in the inhibition make it difficult to tell by manometric methods whether the quinone oxidizes it to disulphide, or reacts to form a thiazole–quinone compound.

Polyvinylpyrrolidone. Polyvinylpyrrolidone has been used to preserve fruit mitochondria during their isolation from tissues rich in polyphenols (Jones & Hulme, 1961; Hulme, Jones & Wooltorton, 1964). There is some uncertainty as to how it acts, but recent evidence suggests that it inhibits apple *o*-diphenol oxidase (Harel, Mayer & Shain, 1964). In agreement with this suggestion is its inhibition of the initial rate of oxidation of chlorogenic acid by tobacco-leaf extracts (Fig. 4). Over the same concentration range it had no effect on the complete oxidation of small amounts of chlorogenic acid; it is therefore unlikely to act either by absorbing chlorogenic acid or the quinones derived from it.

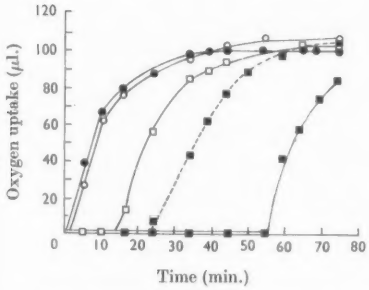


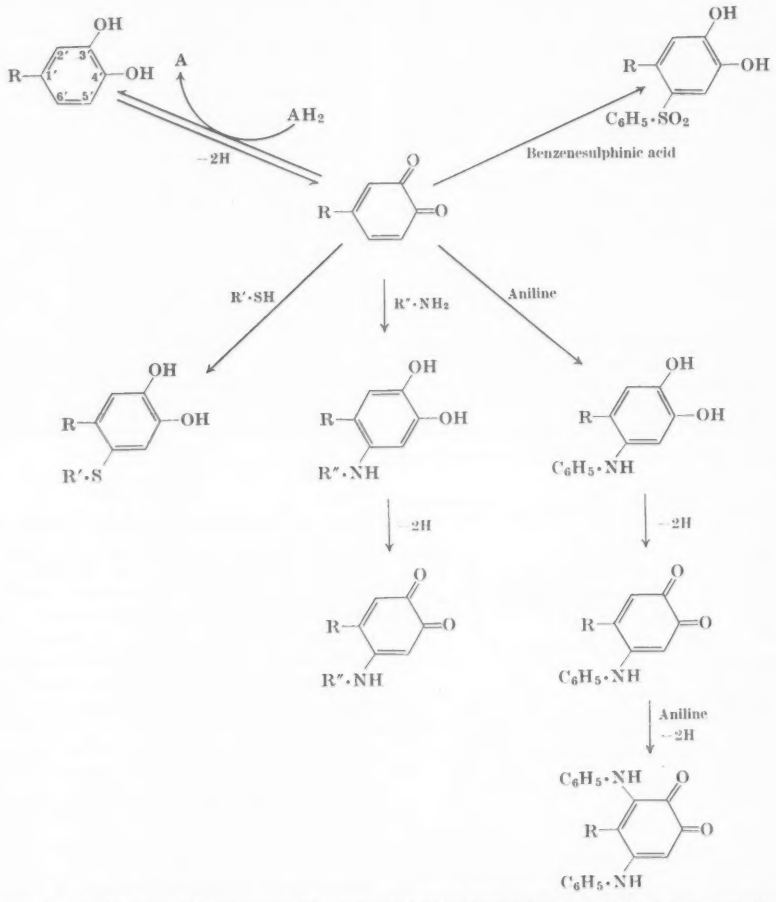
Fig. 8. Delay produced in the action of tobacco-leaf *o*-diphenol oxidase by 2-mercaptobenzothiazole. The enzyme (20µg. of protein N) oxidized chlorogenic acid (24µmoles) at pH 6·8, in the presence of 2-mercaptobenzothiazole of the following concentrations: 0 (●); 0·033mM (○); 0·066mM (□); 0·1mM (■). The broken line shows the course of the oxidation in the presence of 0·1mM-inhibitor at pH 8·2.

DISCUSSION

The effect of benzenesulphinic acid on the oxidation of chlorogenic acid by tobacco-leaf *o*-diphenol oxidase leaves no doubt that the first step in the oxidation is the formation of a quinone with an oxygen uptake of 1 atom/mol. formed. The fate of the quinone is less certain. It may well undergo reactions (2)–(4), analogous to those postulated for β-naphthaquinone in aqueous solution (Fieser & Peters, 1931) and for *o*-benzoquinone formed during the oxidation of catechol by mushroom *o*-diphenol oxidase (Wagreich & Nelson, 1938; Mallette, 1950). The purple compound that appears when proline is present during the oxidation may indicate that a hydroxyquinone is formed (James *et al.* 1948). However, a more likely view is that this pigment is derived directly from the initial quinone rather than a hydroxyquinone (Jackson & Kendal, 1949; Hess, 1958).

Assuming this sequence of reactions to occur, there are two obvious differences from the reactions involved in the oxidation of catechol. First, the hydration of the quinone derived from chlorogenic

acid is much less sensitive to pH than is the hydration of *o*-benzoquinone; pH values below 6 failed to resolve the oxidation of chlorogenic acid into fast and slow components as they do for the oxidation of catechol (Dawson & Nelson, 1938*b*; Jackson & Kendal, 1949). Secondly, the reactions occur less completely, so that the final oxygen uptake is less than 2 atoms/mol. of chlorogenic acid and only approaches this value in dilute solutions of chlorogenic acid (Table 3). This may be because some of the enzymically produced quinone reacts with unchanged chlorogenic acid, especially when this is present in excess, and so prevents its oxidation. An analogous reaction occurs between *o*-benzoquinone and catechol (Dawson & Nelson, 1938*a*) although it apparently has little effect on the amount of oxygen consumed when catechol oxidation is followed manometrically. Alternatively, quinone may be removed from the reaction sequence by the type of quinone–quinone condensation that occurs in concentrated solutions of β-naphthaquinone (Fieser & Peters, 1931).



Scheme 1. Some of the probable reactions of the quinone derived from chlorogenic acid. R represents the quinic acid-containing portion of the chlorogenic acid molecule [·CH:CH·CO_2·$C_6H_7(OH)_3(CO_2H)$]; R′·SH represents thiols such as diethyldithiocarbamate, ethyl xanthate and cysteine; R″·NH_2 represents amino acids such as glycine, cystine and possibly cysteine; AH_2 represents reducing compounds such as ascorbate, thioglycollate and possibly the other thiols tested. Proline and sarcosine react like amino acids with primary amino groups. The possible formation of anil compounds from aniline is not shown.

The quinone derived from chlorogenic acid reacts very much like simpler quinones (see Mason, 1955) and is readily substituted by $-NH_2$, $>NH$, $>CH_2$ and $-SH$ groups. By analogy with the simpler quinones, the most likely position for substitution is the 6′-position of the quinone ring (see Scheme 1); the other likely position (1′) is already occupied by the quinic acid side chain. The proximity of this bulky side chain might be expected to hinder some substitutions at the 6′-position. It would explain the lack of reaction with indole, and possibly also with thioglycollate.

The compounds probably formed from the quinone are shown in Scheme 1. The products of the reaction between the quinone and benzenesulphinic acid or barbiturate, probably 6′-derivatives of chlorogenic acid, are stable and without much colour in neutral solution. The only oxygen absorbed in their formation (1 atom/mol.) is that necessary to form the quinone from chlorogenic acid. The corresponding derivatives of glycine and proline are, however, further oxidized to substituted quinones, absorbing an additional atom of oxygen/mol. and producing intense colours. This also happens to the aniline derivative; but the 6′-anilidoquinone is apparently further substituted by aniline, possibly in the 2′-position, and reoxidized to a dianilidoquinone. A similar dianilidoquinone is thought to be formed from at least one other substituted quinone (Mason, 1955). Disubstitution of the quinone nucleus by indol-3-ylacetic acid probably explains the large oxygen consumption that occurs in the presence of this compound.

The reactions between the quinone of chlorogenic acid and thiols do not involve the uptake of additional oxygen nor do they produce intense colours. The main products are therefore likely to be monosubstituted chlorogenic acid compounds (Scheme 1). But both manometric and chromatographic information suggests that additional reactions occur. The increased oxygen absorption, most noticeable with small concentrations of thiols, may be due to the SH groups reducing the quinone to chlorogenic acid, a reaction that may also occur with thioglycollate. Alternatively, it may be due to some of the monosubstituted dihydric phenol being oxidized by an excess of quinone and then further substituted by thiol. Although this seems less likely, it would explain the formation of two chromatographically distinct products from xanthate. The range of trace products formed from cysteine, however, may be because not only cysteine, but also cystine derived from it, react with quinone via amino groups. Glutathione, which would also be expected to react with quinones through its NH_2 and SH groups, gives two distinct products when it is present during the oxidation of catechol (Mason, 1955); a colourless one when glutathione is in excess, and a purple one when catechol is in excess. Glutathione added to apple tissue oxidizing chlorogenic acid gives rise to 'a whole series of fluorescent compounds' (Hulme, 1958).

All the sulphur-containing compounds tested, except thioglycollate, interfere with polyphenol oxidation by inhibiting the enzyme and also by combining with the quinone product. These two effects explain why xanthate can be used to prepare infective extracts from tobacco leaves infected with cucumber mosaic virus (Pierpoint & Harrison, 1963) at concentrations that do not completely inhibit the leaf *o*-diphenol oxidase. These two effects also explain the reactivation of *o*-diphenol oxidase inhibited by sulphur-containing compounds: traces of quinone react with, and so remove, the inhibitor. In the present work this reactivation has been observed with ascorbate, which is almost certainly oxidized by the quinone, and with 2-mercaptobenzothiazole, which is also probably oxidized (J. K. Palmer, personal communication). The reactivation of insect *o*-diphenol oxidase inhibited by thiourea is more likely to involve the formation of thiourea–quinone complexes (Wojtczak & Chmurzynska, 1960).

Diethyldithiocarbamate and potassium ethyl xanthate have been used as selective inhibitors to implicate copper-containing enzymes in metabolic processes of plants (see James, 1953). In addition to the recognized difficulties of this technique, two new ones appear if inhibitions *in vivo* of *o*-diphenol oxidase can be quickly reversed by accumulating quinones, and if the two inhibitors affect quinone-dependent as well as copper-dependent systems. Thus James & Boulter (1955) found that the respiratory system of 7-day-old barley roots is sensitive to diethyldithiocarbamate, and concluded that a copper-dependent enzyme, probably ascorbic oxidase, was the terminal oxidase. But quinones are now known to be involved in cytochrome electron-transport systems; the possibility that some of them are not fully substituted and so would react with diethyldithiocarbamate is an alternative explanation that ought to be borne in mind.

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Biochem. J. (1966) **98**, 581

The Action of Corticosteroids on Proteolysis

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(*Received* 23 *August* 1965)

1. The corticosteroids cortisol, cortisone and corticosterone were tested for their ability to affect the hydrolysis of serum albumin, insulin and oxyhaemoglobin incubated with trypsin, chymotrypsin, papain and pepsin. 2. Corticosteroids stimulated the hydrolysis of albumin and oxyhaemoglobin with trypsin between 10% and 200% and inhibited the hydrolysis of insulin by 15% (steroid/substrate molar ratio, 5:1). 3. The degree of stimulation of proteolysis for a given substrate depended on both the nature of the steroid and the protease. Corticosterone did not increase the activity of papain and pepsin with any of the substrates tested. 4. Corticosterone stimulated (fivefold) the denaturation of oxyhaemoglobin measured spectroscopically in 2·4% (w/v) sodium hydroxide. Small changes in the absorption spectrum of haemoglobin solutions were also noted at pH 7·8 without a marked change in the basic properties of haemoglobin. 5. With regard to the action of corticosterone on the activity of trypsin, the lack of stimulation when benzoylarginine amide was used as a substrate, the lowering of the stimulation on prior heat denaturation of haemoglobin and the high temperature coefficient for stimulation suggest that the steroid resulted in improved access of the protease to susceptible bonds of the substrate.

Previous experiments indicated that corticosteroids enter cells by diffusion and are then concentrated by adsorption (Bellamy, 1963*a*). Some of the cellular steroids that are strongly attached to insoluble cell proteins are not in diffusion equilibrium with the extracellular phase. Further, a large fraction of bound steroids is non-exchangeable (Bellamy, Phillips, Jones & Leonard, 1962; Bellamy, 1963*b*). Questions arising from these results relate to the biochemical properties of non-enzymic steroid–protein complexes and the mechanism whereby strongly adsorbed steroids are removed from the cell. In this connexion, the following work was undertaken to determine whether corticosteroids could influence protein turnover by affecting the enzymic hydrolysis of various receptor proteins. From the results a model system was defined in which steroids stimulated proteolysis in a highly specific way by interacting with the substrate.

MATERIALS AND METHODS

Proteins and enzymes. Horse oxyhaemoglobin (referred to below as 'haemoglobin') was prepared by Dr M. H. Smith, Department of Biochemistry, University of Sheffield, by the method of Keilin & Hartree (1935), precipitated with ethanol and stored at below 0°. The precipitate was dissolved as required in deionized water and dialysed overnight against deionized water at 10°. Solutions contained between 1 and 2% (dry wt.) of haemoglobin and were stored at 5° for not more than 3 weeks. Bovine serum albumin (fraction V) was obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), and crystalline ox insulin from British Drug Houses Ltd. (Poole, Dorset).

Trypsin (type I), chymotrypsin (type II) and pepsin (crystallized) were obtained from Sigma Chemical Co. Papain was obtained from British Drug Houses Ltd.

Absorption spectra and haemoglobin denaturation. The absorption spectrum of haemoglobin solutions was recorded between 400 and 650 mμ with a Beckman model DB spectrophotometer coupled with a Varicord model 43 recorder. Denaturation was determined by the methods of Brinkman, Wildschut & Wittermans (1934) and Rossi-Fanelli, Azzone & Mondovi (1955) from the increase in extinction at 615 mμ in a 1 cm. light-path (Manwell, 1959). The extent of denaturation was obtained by comparison with the maximum change (2·9-fold increase) in the extinction at 615 mμ (Manwell, 1959), obtained 15 min. after the addition of 0·1 vol. of 60% (w/v) NaOH to a 0·1% solution of haemoglobin in deionized water at 25°.

Measurement of proteolytic activity. Proteases were dissolved immediately before use in an appropriate buffer solution, so that when diluted 1:10 in the protein substrate solution the weight ratio of substrate to enzyme was not less than 100:1. Samples of the medium were removed and mixed with 0·1 vol. of 50% (w/v) trichloroacetic acid. After centrifuging, 0·1 ml. of the supernatant solution was added to 3·0 ml. of water, and 1·0 ml. was used for the estimation of amino nitrogen by the method of Rosen (1957). Corticosteroids (L. Light and Co. Ltd., Colnbrook, Bucks.) were

added dissolved in ethylene glycol. The steroid/protein ratios were calculated on a molar basis assuming mol.wt. 66000 for horse oxyhaemoglobin and 50000 for bovine serum albumin.

Amidase activity of trypsin. A 0·1 ml. portion of trypsin solution (1 mg./ml. in 0·2 M-sodium phosphate buffer, pH 7·8) was added to 0·8 ml. of a 3% solution of sodium benzoyl-L-arginine amide hydrochloride (British Drug Houses Ltd.) at 37°, with and without steroid dissolved in 0·1 ml. of ethylene glycol. After the incubation 0·1 m^1. of 50% trichloroacetic acid was added and the mixture centrifuged. The ammonia content of 0·5 ml. of clear supernatant solution was determined colorimetrically, after microdiffusion (Conway, 1947), with Nessler's reagent (for details see Bellamy & Jones, 1961).

RESULTS

Hydrolysis of albumin by trypsin. Trypsin had little action on native bovine serum albumin, and to obtain a convenient rate of hydrolysis it was necessary to increase the albumin concentration to about 4% (Fig. 1). Even at this concentration less than 1% of the albumin was hydrolysed in 4 hr. at 37°. The addition of corticosterone increased the initial rate of proteolysis (Fig. 2). A 10% stimulation was obtained with as little as 0·36 mM-steroid, equivalent to a steroid/protein molar ratio about 1:2; at a 4:1 molar ratio a 60% stimulation was obtained. The greatest effect of the steroid was obtained in the initial stages of incubation (Fig. 3), and albumin treated with trypsin in the absence of corticosterone was eventually degraded to the same extent (about 4% of the protein hydrolysed in 24 hr.).

This effect of corticosterone was markedly dependent on temperature. A temperature decrease from 37° to 27°, which diminished proteolysis by 50%, completely abolished the action of the steroid.

A range of compounds of biological origin with widely different properties was tested to examine the specificity of the action of corticosterone on trypsin hydrolysis. Unfortunately, most of the compounds were not soluble in glycol to the same extent as corticosterone and only Strophanthin G

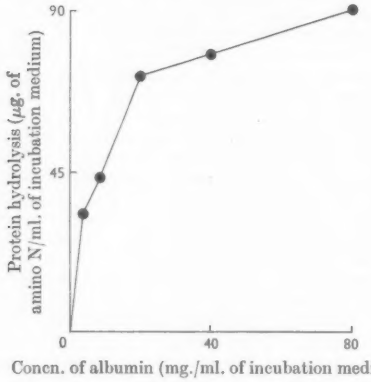

Fig. 1. Effect of albumin concentration on the activity of trypsin. Solutions of serum albumin in water (0·8 ml.) were incubated at 37° for 4 hr. with 0·1 ml. of trypsin (5 mg./ml. in 0·2 M-sodium phosphate buffer, pH 7·8) and 0·1 ml. of ethylene glycol. The reaction was stopped by the addition of 0·1 ml. of 50% trichloroacetic acid, the precipitate was centrifuged down and the amino nitrogen content of the supernatant solution was estimated as described in the text.

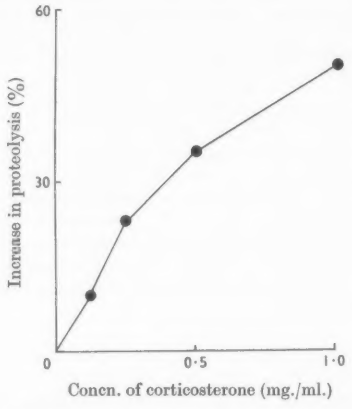

Fig. 2. Effect of corticosterone concentration on the hydrolysis of albumin by trypsin. Incubations were carried out as described in Fig. 1 with 40 mg. of albumin/ml. Various amounts of corticosterone in ethylene glycol (0·1 mg./ml. of incubation medium) were added and the percentage increase in amino nitrogen was determined.

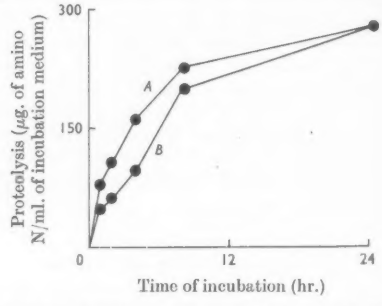

Fig. 3. Effect of corticosterone on the hydrolysis of albumin by trypsin. Incubations were carried out as described in Fig. 2 with (*A*) and without (*B*) corticosterone (1·0 mg./ml. of incubation medium). Samples were removed at intervals and the amino nitrogen content was determined.

remained in solution throughout the incubation period (Table 1). Under these conditions testosterone, β-carotene and thyroxine gave a marked stimulation of proteolysis, although they were not as effective as a similar quantity of corticosterone (Table 1).

Effect of corticosteroids on the enzymic hydrolysis of other proteins. Trypsin had hardly any action on haemoglobin, particularly after the first hour of incubation (Table 2). In contrast, the addition of corticosterone, for example (steroid/protein ratio 30:1), resulted in an increased rate of proteolysis that was maintained for 4hr. (Table 2). This gave a 4–5-fold increase in protein breakdown during the last 3hr. of incubation. A similar but smaller effect was observed with bovine serum albumin but not with insulin (Table 2). When haemoglobin solution was heated at 100° for 2min. and then cooled, before adding trypsin, the rate of protein hydrolysis was increased almost 100-fold. After this treatment, however, corticosterone (steroid/protein ratio 30:1) produced only a 14% increase in trypsin activity. Corticosterone did not stimulate the hydrolysis of benzoylarginine amide by trypsin (Table 2).

Corticosterone was the most effective corticosteroid in stimulating the hydrolysis of albumin by trypsin. Cortisone was almost as effective, whereas cortisol had less than half the action of corticosterone (Table 3). When haemoglobin was used as a substrate corticosterone again gave the greatest stimulation. With this substrate, however, cortisone was the least effective compound. It was also found that besides a marked steroid- and substrate-dependence the type of response was also influenced by the nature of the protease. For example, although papain and trypsin gave similar rates of proteolysis under the same incubation conditions,

Table 1. *Effect of various compounds on the hydrolysis of albumin by trypsin*

A solution or suspension of a range of compounds in glycol (1·25mg./0·1ml.) was incubated with 0·8ml. of 5% albumin and 0·1ml. of trypsin (5·0mg./ml. of 0·2M-sodium phosphate buffer, pH7·8) for 1hr. Each experiment was carried out in duplicate. The rate of albumin hydrolysis was compared with that obtained with glycol alone by using the method described in the text.

Compound	Proteolysis (μg. of amino N/ml. of incubation medium)		Mean change in proteolysis (%)
—	50·0	59·6	—
Corticosterone	98·7	97·3	+89
Testosterone*	89·8	77·4	+52
Thyroxine*	76·4	72·0	+40
β-Carotene*	71·6	76·6	+40
Oestradiol*	64·2	63·7	+17
Bilirubin*	64·3	59·6	+13
Cholesterol*	63·5	61·9	+14
Strophanthin G	56·5	54·2	−1

* Ppt. present during the incubation.

Table 3. *Relative activation of haemoglobin and albumin by corticosteroids*

Incubations were carried out as described in Table 2 for 4hr. Relative proteolysis is expressed as the activity of trypsin in the presence of steroid divided by the activity with glycol alone. Each value is the mean ±S.E.M. from five measurements.

Steroid added	Relative proteolysis	
	Albumin	Haemoglobin
Cortisol	1·25±0·04	1·35±0·02
Cortisone	1·54±0·03	1·23±0·03
Corticosterone	1·63±0·04	2·19±0·04

Table 2. *Effect of corticosterone on the proteolytic and amidase activities of trypsin*

Proteolysis. Incubations were carried out at 37° with 0·8ml. of 1% protein solution, 0·1ml. of ethylene glycol with (C) and without (N) corticosterone (1·25mg.) and 0·1ml. of trypsin solution (1mg./ml. in 0·2M-sodium phosphate buffer, pH7·8. The reaction was stopped by the addition of 0·1ml. of 50% trichloroacetic acid and the amino nitrogen was determined by the method of Rosen (1957). Amidase activity. Incubations were carried out as above except that 0·8ml. of 3% sodium benzoylarginine amide hydrochloride in deionized water was used in place of the protein solutions.

Substrate	Proteolysis (μg. of amino N/ml. of incubation medium)						Amidase activity (μg. of NH_3/ml. of incubation medium) Benzoylarginine amide	
	Haemoglobin		Serum albumin		Insulin			
Time (hr.)	N	C	N	C	N	C	N	C
0	1·05	1·05	7·72	7·72	7·01	7·01	8·78	8·78
1	6·32	9·60	31·5	40·5	—	—	66·2	55·6
4	11·9	33·0	59·7	89·8	23·9	20·5	—	—

corticosterone decreased the rate of albumin hydrolysis with papain by almost 25%. The effect on insulin hydrolysis was the same as with trypsin (Table 4; cf. Table 2). Pepsin gave very high rates of proteolysis but corticosterone had no action on the hydrolysis of either substrate (Table 4). Corticosterone produced effects with chymotrypsin similar to those obtained with trypsin.

Corticosterone and haemoglobin denaturation. In view of the strong possibility that corticosterone stimulated proteolysis by a process of denaturation of the substrate, this steroid was tested for its ability to denature haemoglobin in alkaline solution. It was found that the addition of corticosterone to haemoglobin solution increased the rate of denaturation in 2·4% (w/v) sodium hydroxide (Table 5). With a steroid/protein ratio 300:1 almost all of the haemoglobin was denatured in about 2min. compared with about 17% in the absence of corticosterone. The effect was dependent on the quantity of added steroid and with a corticosterone/haemoglobin ratio about 3:1 there was only a 10% change in the rate of denaturation.

Preincubation of haemoglobin with corticosterone (steroid/protein ratio 300:1) at pH7·8 brought about a 10% decrease in the extinction at 580mμ and an increase of 45% at 615mμ. These changes followed first-order kinetics and took about 2hr. to reach completion at 37°. The spectrum between 650 and 450mμ still had the same general form of oxyhaemoglobin. It was also found that the basic properties of the haemoglobin, as judged by its ability to be reversibly deoxygenated and to combine with carbon monoxide and cyanide, had not changed. The rate of alkaline denaturation of

Table 4. *Action of corticosterone on the hydrolysis of albumin and insulin by various proteases*

Papain. Incubations were carried out at 37° with 0·8ml. of 5% protein solution, 0·1ml. of ethylene glycol with (C) and without (N) corticosterone (1·25mg.) and 0·1ml. of papain (5mg./ml. of 0·2M-sodium phosphate buffer, pH7·8). Pepsin. Incubations were carried out as for papain except that the substrate concentration was 0·5% and the enzyme solution was 0·5mg./ml. of 0·2M-glycine–HCl buffer, pH2·2. Each reaction was stopped after 4hr. at 37° by the addition of 0·1ml. of 50% trichloroacetic acid and the amino nitrogen was determined as described in the text. Each estimation was carried out in duplicate.

	Proteolysis (μg. of amino N/ml. of incubation medium)			
	Albumin		Insulin	
	N	C	N	C
Papain	18·5	14·6	24·0	22·6
	17·9	13·3	24·4	23·9
Pepsin	81·2	81·8	131	122
	83·2	83·5	126	129
Chymotrypsin	61·5	77·7	13·3	12·0
	63·1	73·8	11·5	10·8

Table 5. *Effect of corticosterone on the rate of alkaline denaturation of haemoglobin*

Solutions were prepared containing 2·4ml. of 0·12% haemoglobin and 0·3ml. of sodium phosphate buffer, pH7·8. Ethylene glycol with and without dissolved corticosterone was added as indicated (0·3ml.). Incubations were carried out for 130min. at 37°. The rate of alkaline denaturation was measured as described in the text after the addition of 0·1ml. of 60% NaOH to 2·5ml. of haemoglobin solution at 24°. In the unincubated samples 60% NaOH was added 3min. after adding ethylene glycol. The extent of denaturation was calculated assuming that the extinction observed 3min. after the addition of 0·1ml. of 60% NaOH to unincubated haemoglobin (with 7·5mg. of corticosterone) was the maximum value for denatured haemoglobin.

	Denatured haemoglobin (%)					
	Not preincubated		Preincubated			
Corticosterone added (mg.).....	0	3·2	0	0·032	0·32	3·2
Time after addition of NaOH (min.)						
0	0	0	0	0·90	2·24	11·9
1	10·5	77·4	18·9	22·0	38·6	84·1
2	16·6	95·6	25·0	28·0	43·5	92·0
3	21·4	100	29·2	32·6	48·5	92·6
4	25·6	—	33·0	36·0	53·3	92·4
6	32·4	—	39·0	41·5	58·2	92·6
8	35·8	—	42·0	44·7	59·7	—
10	38·2	—	44·9	46·6	62·0	—
12	39·9	—	46·5	49·5	64·0	—
15	42·6	—	48·3	50·7	65·0	—

haemoglobin preincubated with 0·032mg. of corticosterone did not differ greatly from that of the control, taking into account that preincubation with ethylene glycol alone apparently made the haemoglobin more labile under alkaline conditions (Table 5). With 3·2mg., preincubation decreased the degree of stimulation by about 40%. However, the spectrum of the alkaline-denatured preincubated haemoglobin differed slightly from that of haemoglobin denatured in the presence of corticosterone without a preincubation period. In particular, the maximal extinction change at 615mμ was almost 10% greater.

DISCUSSION

In general, steroid hormones show a greater tendency to interact with proteins than with lipids (Bischoff, Stauffer & Gray, 1954; Bellamy, 1963*b*). The adsorption of steroids to plasma proteins has been investigated in great detail (Antoniades, 1960) and it has been suggested that steroids are attached to a wide range of proteins by hydrogen bonds (Westphal, 1961). This probably leads, in turn, to the breakage of intramolecular bonds linking peptide chains, and the process may be interpreted as a form of denaturation. In keeping with this the effect of steroids on individual enzymes is generally one of inhibition (Leiberman & Teich, 1953; Tomkins & Maxwell, 1963; Table 4 for trypsin with benzoylarginine amide as substrate), although a particular steroid may both stimulate and inhibit a given enzyme depending on the steroid/protein ratio (Dirscherl, 1962; Jones & Bellamy, 1964). However, steroids might be expected to produce alterations in the structure of proteins without changing their basic biological properties. With respect to corticosterone–haemoglobin mixtures, the formation of a new type of protein structure may be inferred from the different spectrum, the increased tendency to denature in alkaline solution and the ease with which the steroid–protein mixture was hydrolysed with trypsin. The basic properties of the haemoglobin, however, were not changed appreciably at a concentration of steroid that stimulated proteolysis.

When considering the action of corticosteroids on the proteolytic activity of trypsin, the substrate-specificity, taken together with the absence of stimulation with benzoylarginine amide as substrate, argues against a direct activation of the enzyme. Further, the small effect on denatured haemoglobin, the spectral changes on treatment of this protein with corticosterone and the marked temperature-dependence of the stimulation are strongly in favour of an interaction with the substrate that results in an improved access of the protease to susceptible bonds.

Changes in protein tertiary structure seem to occur with a wide range of molecules (allosteric modifiers; Bitensky, Yielding & Tomkins, 1965) and it would not be surprising for non-polar compounds in general to change proteins sufficiently to affect the rate of enzymic hydrolysis. The question is whether the present observations throw light on the specific action of corticosteroid hormones. In this connexion, there can be little doubt that the system steroid–receptor protein–protease, with two highly variable protein components, offers, theoretically, an ideal situation for bringing about an increase in the turnover of specific protein receptors. Therefore, investigations into intracellular mechanisms related to the effect of cortisol on protein degradation (Hoberman, 1950; Russell, 1951) should take into account the effects of a substrate level interaction of the steroid as well as the likelihood of a direct activation of cathepsins.

Turning to the question of the removal of protein-bound steroids from the cell, there are two possibilities to be considered. The steroid may be loosely bound and in equilibrium with extracellular steroid. This appears to be true for some of the biologically active cortisol in liver (Bellamy & Leonard, 1964). If, on the other hand, the steroid is strongly bound in a tissue such as muscle (Bellamy, 1963*b*), with no possibility of metabolism, the hormone would have a chance of leaving the cell only on turnover of the receptor protein. The present work opens up the possibility that, in the latter situation, attachment of the steroid may shorten the life span of the receptor.

It is appreciated that the concentration of steroid required to produce the effects was at least 100 times normal blood concentrations. This was a consequence of the high concentration of substrate necessary for an accurate measurement of protein hydrolysis. Changes in proteolysis were obtained with a steroid/substrate protein molar ratio between 1:1 and 5:1, and at the moment there is no reason to suppose that the intracellular relationship between steroid and receptor renders this relationship invalid.

The work was supported by grants to the Department of Zoology, University of Sheffield, from the Agricultural Research Council and the Science Research Council. The authors are indebted to Dr M. H. Smith, Department of Biochemistry, University of Sheffield, for the gift of horse oxyhaemoglobin and for his help with the spectroscopic analysis of haemoglobin.

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Electrophoretic and other Studies on Haem Pigments from *Rhodopseudomonas palustris*: Cytochrome 552 and Cytochromoid *c*

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(*Received* 29 *July* 1965)

1. Cytochrome 552 and cytochromoid *c* were extracted from *Rhodopseudomonas palustris* cells, purified and obtained in crystalline form. 2. Extinction ratios and amino acid compositions of the two pigments are reported. 3. When subjected to starch-gel electrophoresis in borate buffer, pH 8·8, each pigment migrated towards the cathode; oxidized cytochromoid *c* migrated more rapidly than its reduced form. 4. By a determination of electrophoretic mobilities in buffers of I 0·1 by using the moving-boundary method, the isoelectric point of cytochrome 552 was found to be at pH 10·6 and that of cytochromoid *c* at pH 9·7. 5. As obtained, cytochrome 552 was non-autoxidizable; cytochromoid *c* was autoxidizable but became considerably less so on alkaline treatment. 6. Discussion of the results includes a consideration of the isoelectric points of the pigments in terms of their amino acid composition.

When grown anaerobically in the light the purple photosynthetic bacteria contain appreciable concentrations of a *c*-type cytochrome and the related cytochromoid *c* (Kamen, 1963*a*; Porra & Lascelles, 1965). For this study, the two representative haemoproteins were prepared from *Rhodopseudomonas palustris* in crystalline form; as far as we know, the cytochromoid from this source has not previously been obtained as crystals.

During starch-gel electrophoresis at pH 8·8 both pigments migrated towards the cathode. Isoelectric points well on the alkaline side of neutrality are unusual, as most bacterial cytochromes have an isoelectric point less than 7 and all of the *c*-type cytochromes and cytochromoids from the photosynthetic bacteria so far examined have had isoelectric points less than 7 (Dus & Kamen, 1963). Further, Morita (1960), using a crystalline preparation of *Rps. palustris* cytochrome 552, has estimated the isoelectric point as 7·7, from a determination of electrophoretic mobilities by the moving-boundary method. In view of the well-known difficulties attendant on comparison of results from gel electrophoresis with those from the moving-boundary method it was decided to determine the electrophoretic mobilities of each pigment over a range of pH values in the Tiselius-type electrophoretic apparatus. To characterize the pigments by comparison with published information, spectrophotometric and other data obtained are also reported. de Klerk, Bartsch & Kamen (1965) reported a cathodic migration of the *Rps. palustris* haemoproteins cytochrome 552 and cytochromoid *c*, when these were subjected to starch-gel electrophoresis at pH 8·9.

A preliminary report of this work has been made (Henderson & Nankiville, 1965).

MATERIALS

Cultures. The strain of *Rps. palustris* used in this study was kindly provided by Dr M. D. Kamen, University of California, San Diego, Calif., U.S.A., and was originally strain no. 2137 from the collection of Dr C. B. van Niel, Stanford University, Calif., U.S.A. The organism was cultivated photosynthetically under aseptic conditions in what was essentially the medium used by Cohen-Bazire, Sistrom & Stanier (1957) for the cultivation of *Rhodospirillum rubrum*. The biotin used in the salt solution by these workers was replaced by 0·1% (w/v) Difco yeast extract; also, 0·1% (w/v) L-glutamic acid and 0·1% (w/v) sodium acetate trihydrate were used instead of the Difco casein hydrolysate. The cultures were grown at 34° in 12l. flasks equipped with cooling coils, under a bank of 300w tungsten lamps, the light–intensity at the culture surface being 600ft.-candles. Oxygen-free nitrogen was bubbled slowly through the cultures. The cells were harvested after they had reached the stationary phase (5–7 days) by use of a Cepa Schnell continuous-flow centrifuge (model LE I; Carl Padberg, Düsseldorf, Germany) operated at 32000*g* (25000rev./min.) and a flow rate of 10–12l./hr. They were then washed twice by resuspension in one-tenth the culture volume of water at 4°, followed by centrifugation for 30min. at 1750*g* (International model PR 2, 850A head). After

washing, the cells were freeze-dried and stored over silica gel at 4°.

Reagents. All chemicals were of analytical-reagent grade except for tris buffer, which was British Drug Houses Ltd. (Poole, Dorset) laboratory-reagent grade.

Cytochrome c. This was prepared from ox-heart muscle by the method of Keilin & Hartree (1952) and contained 0·33% of iron.

METHODS

Spectrophotometry

Absorption spectra were obtained by means of a Cary model 14 recording spectrophotometer. A Zeiss model PMQ II spectrophotometer was also used for extinction determinations. Reduced preparations were obtained by addition of a slight excess of solid $Na_2S_2O_4$ to the pigment sample in buffer. Oxidized preparations were obtained by addition of a slight excess of 3mM-$K_3Fe(CN)_6$ to the pigment sample in buffer followed by dialysis against several changes of the buffer. When dilution was to be avoided, as in the determinations of the percentage reduction, a slight excess of finely powdered solid $K_3Fe(CN)_6$ was added. The percentage reduction of cytochrome 552 present in a partially reduced solution (as was required for the autoxidation experiments) was obtained by substitution in the equation:

$$\text{Reduction (\%)} = \frac{E^{1\,\text{cm.}}_{552} - E^{1\,\text{cm.}}_{552\,(\text{ox.})}}{E^{1\,\text{cm.}}_{552\,(\text{red.})} - E^{1\,\text{cm.}}_{552\,(\text{ox.})}} \times 100$$

where $E^{1\,\text{cm.}}_{552}$ is the extinction of the partially reduced solution, and the other extinction values were obtained from a sample of the solution that was completely oxidized and then completely reduced as above.

Electrophoresis

Starch-gel electrophoresis. This was carried out on horizontal gels with Connaught Laboratories (Toronto, Canada) hydrolysed starch made up in 0·023M-sodium borate buffer, pH 8·8, as described by Smithies (1955). Reduced cytochrome 552 and ox-heart cytochrome *c* were prepared by the addition of a slight excess of $Na_2S_2O_4$ followed by dialysis for 5hr. against several changes of the above borate buffer. Oxidized cytochromes were prepared by addition of a slight excess of $K_3Fe(CN)_6$ followed by dialysis as for the reduced pigments. Cytochromoid *c*, as obtained below, was already fully oxidized; the reduced form was prepared by addition of a slight excess of $Na_2S_2O_4$ immediately before electrophoresis. Electrophoresis was carried out for about 1hr. at 4° and 6v/cm. Amido Black 10B was used for staining.

Moving-boundary electrophoresis. Mobility determinations were carried out with crystalline samples of reduced cytochrome 552 and oxidized cytochromoid *c* in buffers of I 0·1 in the 2ml. cell of a Perkin–Elmer model 38 Tiselius-type electrophoresis apparatus. Buffers were made up as described by Datta & Grzybowski (1961) and checked at the temperature of electrophoresis (1·5°) with a glass electrode. Solutions for electrophoresis were equilibrated by dialysis against the appropriate buffer. Electrophoresis was carried out for 3–4hr. at a potential gradient of about 10v/cm.

Assay methods

Amino acid analysis. A Beckman model 120B amino acid analyser was used. Samples were hydrolysed for 22hr. in 6N-HCl at 110° in sealed evacuated tubes. For cytochrome 552 the results are the means of two separate hydrolyses with duplicate determinations on each hydrolysate. Ammonia was estimated on one such hydrolysate by a Conway-type distillation followed by a modification of the colorimetric procedure of Brown, Duda, Korkes & Handler (1957). Owing to the small amount available, results with cytochromoid *c* are the means of duplicate determinations on one hydrolysate. The ammonia estimation was carried out in the amino acid analyser.

Iron analysis. The method of Drabkin (1941) was used with slight modification (Henderson & Rawlinson, 1956).

Isolation and purification of cytochrome 552 and cytochromoid c

'pH 7 *alumina*'. Alumina (Judex, chromatographic grade) was heated with N-NaOH (80°) for 20min., washed thoroughly with water, and the fraction settling in 3min. retained. The alkaline treatment and washing was then repeated. The alumina was next stirred with N-HCl for 20min. and then washed with water. The pH of the suspension was adjusted to 7 with N-NaOH and, after further washing with water, the alumina was equilibrated to pH 7·0 by repeated washing with 0·1M-sodium phosphate buffer, pH 7·0. Columns of the alumina were washed free from fines with 0·01M-sodium phosphate buffer, pH 7·0; this was checked by ensuring that $E^{1\,\text{cm.}}_{280}$ of the effluent was not more than 0·003. Immediately before application of the sample, the column was washed with three times the alumina-volume of water.

'pH 7·5 *resin*'. Amberlite CG-50 (100–200 mesh) was stirred with 5% (w/v) KOH at 60° for 20min., then thoroughly washed with water, and the fraction settling in 15sec.–1min. retained. This treatment was repeated and the resin was then stirred with 5% (v/v) H_2SO_4 at 60° for 10min. After washing with water, the pH of the suspension was adjusted to 7·5 with 5% (w/v) KOH, after which it was washed with water. To achieve complete equilibration to pH 7·5 it was necessary to wash repeatedly with 0·1M-potassium phosphate buffer, pH 7·5. Columns of this resin were tested for fines and washed with water immediately before application of the sample, and in a manner similar to that for the 'pH 7 alumina' columns.

'pH 8·0 *resin*'. The column was poured with 'pH 7·5 resin', then 2M-ammonium phosphate buffer, pH 8·0, was passed through the column: at least 50ml. for a 10cm.× 1cm. column. The column was then washed with water and the effluent checked for fines and proteins as described above.

Disruption of cells and extraction of pigments. Disruption of the cells was carried out with an MSE 500w, 20 kcyc./sec. ultrasonic generator. Freeze-dried cells (25g.) were suspended in 250ml. of 0·1M-tris buffer, pH 8·0, and the suspension was separated into two approximately equal portions. Disruption of one portion was carried out for 1min. in an ice bath; then, while this was being stirred in an ice bath, the second portion was similarly disrupted for 1min. This procedure was maintained for a disruption time of 30min. for each portion. The resultant mixture

was diluted with 100 ml. of tris buffer, pH 8·0, and extracted overnight with continuous stirring. It was then sedimented for 30 min. at 17500*g* (Servall SS1). The supernatant was separated and stored at 4°, and the precipitate was resuspended in 160 ml. of tris buffer, pH 8·0, and disrupted in a similar manner to that described above for a further total disruption time of 60 min. The mixture was again diluted with 100 ml. of tris buffer, pH 8·0, and extracted overnight with continuous stirring. The residue was then removed by centrifugation for 30 min. at 17500*g*. The supernatants from the first and second extractions were combined and dialysed against 15 vol. of water with four changes over 2 days. Fine particles, which formed during dialysis, were removed by centrifugation at 10000*g* for 20 min. to avoid blocking the column at the next stage. In Fig. 1 the release of haemoprotein is compared with time of ultrasonic treatment at pH 8·0.

Separation and crystallization of pigments. The dialysed solution was first chromatographed on a column (17 cm. × 2·5 cm.) of 'pH 7 alumina'. Cytochrome 552 and cytochromoid *c* were adsorbed at the top of the column whereas the bacteriochlorophyll and carotenoids passed through. After being well washed with water, the haemoproteins were eluted with 120 ml. of 2 M-ammonium phosphate buffer, pH 8·0, and the eluate was dialysed against 10 vol. of water with four changes over 2 days. A colourless precipitate that formed during dialysis was removed by centrifugation. The impure mixture of cytochrome 552 and cytochromoid *c* was next applied to a column (17 cm. × 1·5 cm.) of 'pH 7·5 resin'; this left the two pigments adsorbed at the top of the column, which was then washed with water. The washing removed some yellow–brown material, which may have been modified haemoprotein, as it showed a very weak haemochromogen-type spectrum on reduction with $Na_2S_2O_4$. Continued washing resulted in a separation of cytochromoid *c* from cytochrome 552. The oxidized cytochrome 552 was strongly adsorbed as a red zone at the top of the column with a coloured pink front of the less strongly adsorbed reduced form, whereas the cytochromoid *c* separated as a slowly moving brown zone becoming more diffuse as it moved down the column. When it was apparent that most of the cytochromoid *c* had been separated from the cytochrome 552 and while the former was still on the column, the resin that contained the cytochrome 552 was removed by suction as a slurry with water and poured on top of another small column (2 cm. × 1 cm.) of 'pH 7·5 resin'. Cytochrome 552 and cytochromoid *c* were then eluted from their separate columns with 30 ml. of 0·1 M-potassium phosphate buffer, pH 7·5, and the eluates were dialysed against water.

Cytochromoid *c* was further purified by passage through a column (10 cm. × 1 cm.) of 'pH 8·0 resin', which strongly

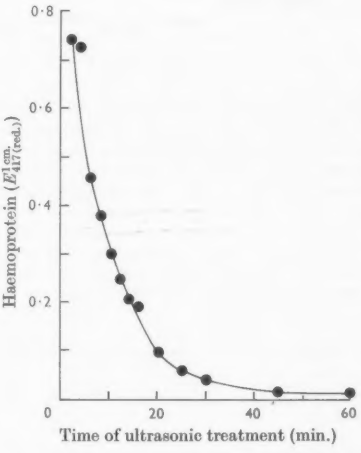

Fig. 1. Plot of release of haemoprotein from *Rps. palustris* against time of ultrasonic treatment. A suspension of 3 g. dry wt. of cells was made in 40 ml. of 0·1 M-tris buffer, pH 8·0; this was kept in an ice bath throughout the ultrasonic treatment. Disruption was carried out for 1 min. periods alternating with 1 min. cooling. At each successive point shown, the suspension was centrifuged at 17500*g* for 15 min. and the supernatant was removed and brought to 20% (w/v) by the addition of solid $(NH_4)_2SO_4$. This treatment precipitated the bacteriochlorophyll and carotenoids and left the haemoproteins in solution. The latter were separated by centrifugation (1500*g*) and estimated by measurement of $E^{1\,cm.}_{417}$ after reduction with $Na_2S_2O_4$.

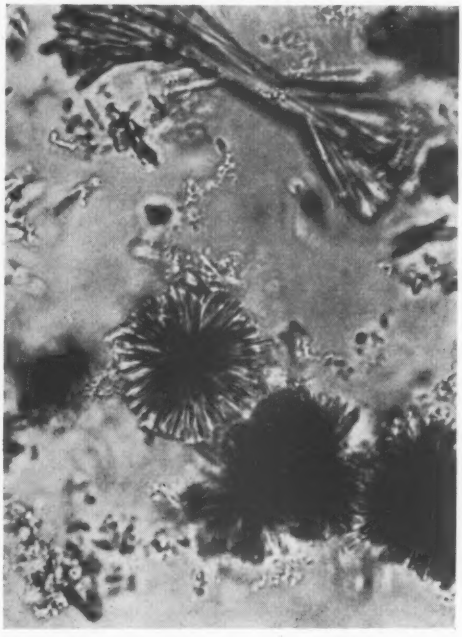

Fig. 2. Crystals of oxidized *Rps. palustris* cytochromoid *c*. Magnification × 725.

adsorbed any remaining cytochrome 552 whereas the cytochromoid *c* was not adsorbed. The cytochromoid *c* was then applied to a column (10 cm. × 1 cm.) of 'pH 7·5 resin', washed well with water and then eluted with 0·1 M-potassium phosphate buffer, pH 7·5. At this stage the following extinction ratios were obtained for the oxidized pigment: E_{642}/E_{283} 0·13 and E_{395}/E_{276} 3·61. Oxidized cytochromoid *c* was obtained in crystalline form by dialysing the above eluate against saturated $(NH_4)_2SO_4$ for 24 hr. (Fig. 2).

Further purification of cytochrome 552 was carried out by adsorption on a column (10 cm. × 1 cm.) of 'pH 8·0 resin', after which it was washed with water to remove any remaining cytochromoid *c*. The cytochrome 552 that was then eluted with 0·1 M-potassium phosphate buffer, pH 7·5, possessed an extinction ratio $E_{552\,(red.)}/E_{275\,(ox.)}$ 1·12. The preparation was obtained in crystalline form at this stage by dialysis of the reduced pigment against 70% saturated $(NH_4)_2SO_4$. The first crystals appeared after 48 hr. as rosettes of red needles; after 8 weeks the rosettes were largely replaced by lanceolate forms and some rectangular plates.

RESULTS

Spectrophotometry. Absorption spectra of both oxidized and reduced forms of cytochrome 552 and cytochromoid *c* were obtained in the visible and ultraviolet regions of the spectrum from solutions in 0·05 M-potassium phosphate buffer, pH 7·0. For cytochromoid *c* the dissociation of the carbon monoxide complex is significant at atmospheric pressure (Kamen, 1963*a*; Bartsch, 1963). Therefore it was prepared in a cuvette equipped with a gassing attachment that permitted the sample to be first flushed with carbon monoxide, and then the pressure raised to 1 atm. above atmospheric pressure. While under this increased pressure, a slight excess of sodium dithionite was introduced from a side arm and the sample examined in the spectrophotometer. The spectra obtained indicated no significant difference from those published by Bartsch (1963) and de Klerk *et al.* (1965). Extinction ratios of the absorption maxima obtained from these spectra are presented in Table 1.

On an iron-content basis, $\epsilon_{552\,(red.)}$ is 23·2 × 10^3 l. mole^{-1} cm.$^{-1}$ for cytochrome 552 at pH 7·0 in 0·05 M-potassium phosphate buffer and this value was used for estimations of concentration. Insufficient cytochromoid *c* was available for determination of ϵ, so in this case concentrations were estimated by using the value of $\epsilon_{435\,(red.)}$, 184 × 10^3 l. mole^{-1} cm.$^{-1}$, given by Bartsch (1963).

Starch-gel electrophoresis. The results of starch-gel electrophoresis in 0·023 M-sodium borate buffer, pH 8·8, of the oxidized and reduced forms of *Rps. palustris* cytochrome 552 and cytochromoid *c* and of ox-heart cytochrome *c* are compared in Fig. 3. Oxidized and reduced cytochrome 552 showed comparable rates of migration towards the cathode whereas reduced ox-heart cytochrome *c* migrated more rapidly towards the cathode than its oxidized form. The oxidized form of cytochromoid *c*

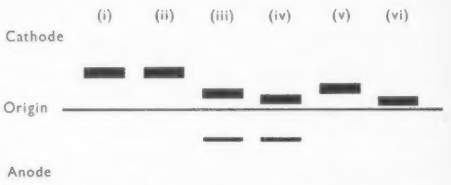

Fig. 3. Starch-gel electrophoresis of *Rps. palustris* cytochrome 552 and cytochromoid *c* and ox-heart cytochrome *c* in borate buffer, pH 8·8. (i) Oxidized cytochrome 552; (ii) reduced cytochrome 552; (iii) reduced ox-heart cytochrome *c*; (iv) oxidized ox-heart cytochrome *c*; (v) oxidized cytochromoid *c*; (vi) reduced cytochromoid *c*. Impurities in the ox-heart cytochrome *c* preparation which migrated to the anode are indicated in (iii) and (iv). Electrophoresis was carried out for 45 min. at 4° and 6 v/cm.; Amido Black 10B was used for staining.

Table 1. *Extinction ratios of crystalline preparations of* Rps. palustris *cytochrome 552 and cytochromoid* c

Cytochrome 552		Cytochromoid *c*			
Wavelength (x mμ)	$\frac{E_x}{E_{527\,(ox.)}}$	Wavelength (x' mμ)	$\frac{E_{x'}}{E_{552\,(red.)}}$	Wavelength (x'' mμ)	$\frac{E_{x''}}{E_{570\,(CO-red.)}}$
Oxidized		Oxidized		CO–reduced	
527	1·00	642	0·277	570	1·00
412	10·5	500	0·98	535	1·19
275	2·20	398	7·88	418	22·2
		283	2·17		
Reduced		Reduced			
552	2·47	552	1·00		
522	1·59	426	8·75		
418	12·8				

migrated more rapidly towards the cathode than the reduced form. Cytochrome 552 migrated more rapidly than reduced ox-heart cytochrome *c*, which migrated at a slightly lower rate than oxidized cytochromoid *c*. Relatively short runs were essential, owing to the tendencies for oxidized ox-heart cytochrome *c* to become reduced during electrophoresis, and for reduced cytochromoid *c* to become oxidized. In each case the respective change in redox state was followed by an increase in the rate of migration of the pigment. Impurities in the ox-heart cytochrome *c*, which are known to migrate towards the anode under these conditions (Henderson & Paléus, 1963), are indicated in Fig. 3.

Moving-boundary electrophoresis. Electrophoretic mobilities of cytochrome 552 in the pH range 4·0–11·1 in buffers of *I* 0·1 are plotted against pH in Fig. 4. The isoelectric point of 10·6 for cytochrome 552 is close to the value 10·65 obtained by Theorell & Åkesson (1941) for ox-heart cytochrome *c*.

Electrophoretic mobilities of cytochromoid *c* in the pH range 5·0–11·1 in buffers of *I* 0·1 are plotted against pH in Fig. 5. As insufficient pigment was available to enable a fresh sample to be taken for each run, each sample was used as far as possible for not more than two runs and then always first at a near-neutral pH value followed by a more alkaline or acid value. Nevertheless, difficulty was experienced in obtaining consistent mobility results at pH 7–8. At extremes of the pH range investigated, these difficulties were not experienced and the mobilities fitted into the expected pattern. Disregarding the equivocal results referred to, the remaining mobilities fit a curve that shows zero mobility at pH 9·7.

Iron contents. A sample of cytochrome 552 with an extinction ratio $E_{552\,(red.)}/E_{275\,(ox.)}$ 1·12 contained 0·33% of iron. This means that the minimum molecular weight is about 17 000. Insufficient cytochromoid *c* was available for assay of iron.

Amino acid compositions. Amino acid analyses of crystalline samples of cytochrome 552 and cytochromoid *c* are presented in Table 2. After hydrolysis there was some dark humin present in each case. When buffer was added to the hydrolysate after drying over potassium hydroxide, the humin was present as a finely divided suspension. After thorough stirring, this material was removed by centrifugation at 1500*g* and samples of the supernatant were taken for column-chromatographic analysis. Decomposition of serine and threonine by 10% (Smith & Stockell, 1954) was allowed for in the results. The cysteine residues covalently bound to the haem are incompletely disrupted under the conditions of hydrolysis (Theorell, 1938) and therefore the values obtained for half-cystine are very probably low. The amino acid compositions of cytochrome 552 and cytochromoid *c* reported are similar to those published by Kamen (1963*b*).

Autoxidation of cytochrome 552 *and cytochromoid* c. A 30 μM solution of cytochrome 552 in 0·05 M-potassium phosphate buffer, pH 7·0, was partially

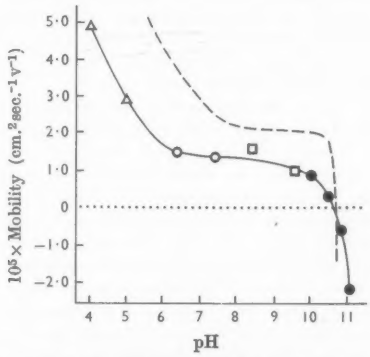

Fig. 4. Plot of electrophoretic mobilities of *Rps. palustris* cytochrome 552 against pH. The buffers, *I* 0·1, were sodium acetate (△), sodium–potassium phosphate (○), tris (□) and glycine (●). All buffers were prepared as described by Datta & Grzybowski (1961). Electrophoresis was carried out for 2–5 hr. at 1·5° and about 10 v/cm. ——, present results; – – – –, curve obtained by Theorell & Åkesson (1941) for ox-heart cytochrome *c*. The plotted mobilities are means from each limb of the cell.

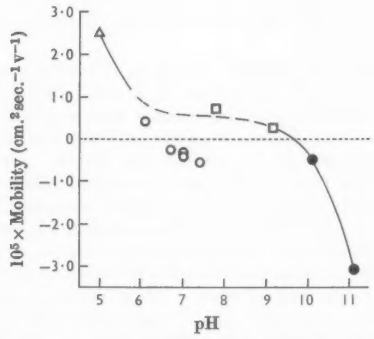

Fig. 5. Plot of electrophoretic mobilities of *Rps. palustris* cytochromoid *c* against pH. The buffers, *I* 0·1, were sodium acetate (△), sodium–potassium phosphate (○), tris (□) and glycine (●). All buffers were prepared as described by Datta & Grzybowski (1961). Electrophoresis was carried out for 1–5 hr. at 1·5° and about 10 v/cm. The plotted mobilities are means from each limb of the cell.

Table 2. *Amino acid analyses of* Rps. palustris *cytochrome* 552 *and cytochromoid* c *and horse-heart cytochrome* c

The cytochromoid *c* results are the means of duplicate determinations on a sample possessing the ratio $E_{642\,(ox.)}/E_{283\,(ox.)}$ 0·10. The cytochrome 552 results are the means of duplicate determinations on two samples each possessing the ratio $E_{552\,(red.)}/E_{275\,(ox.)}$ 1·12. Values have been corrected for 10% destruction during hydrolysis of serine and threonine (Smith & Stockell, 1954).

	Amino acid composition (moles/100 moles)		
	Rps. palustris cytochromoid *c*	*Rps. palustris* cytochrome 552	Horse-heart cytochrome *c* (from Margoliash, Kimmel, Hill & Schmidt, 1962)
Lys	12·1	10·4	18·3
His	0·8	1·5	2·9
NH_3	7·4	17	7·7
Arg	2·1	2·8	1·9
Asp	10·9	12·9	7·7
Thr	5·3	7·3	9·6
Ser	4·4	3·1	0
Glu	9·7	8·0	11·5
Pro	3·7	3·6	3·8
Gly	7·6	9·5	11·5
Ala	17·7	14·6	5·8
CyS (half)	0·9	0·8	1·9
Val	4·3	6·6	2·9
Met	2·2	2·4	1·9
Ile	5·4	1·7	5·8
Leu	8·2	8·0	5·8
Tyr	1·0	2·6	3·8
Phe	3·8	3·9	3·8
Trp	—	—	1·0

reduced with sodium dithionite and then dialysed against three changes of the above buffer. After this treatment the cytochrome was 70% reduced. It was then exposed to air in a test tube and shaken at intervals of several minutes at 24°. Over a period of 10hr. under these conditions, followed by a further 14hr. standing without shaking, there was no detectable change in extinction at 552mμ.

Solutions of 6μM-cytochromoid *c* in 0·05M-potassium phosphate buffer, pH 7·0, were placed in test tubes and reduced with a slight excess of sodium dithionite. The tubes were shaken at intervals of 30sec. and the decrease in extinction at 440mμ was observed. Samples from two different preparations, each possessing the ratio $E_{642\,(ox.)}/E_{283\,(ox.)}$ 0·13 at pH 7·0, were completely oxidized within 5min. Further samples from the above preparations were subjected to alkaline treatments as follows: (i) dialysed against 4mN-ammonia for 6hr. at 4° followed by equilibration against 0·05M-potassium phosphate buffer, pH 7·0; (ii) treated for 15hr. in 0·1M-potassium phosphate buffer, pH 11·6, at 4° followed by neutralization and equilibration against 0·05M-potassium phosphate buffer, pH 7·0. Each of the alkali-treated samples required 20min. for complete oxidation under the conditions used for the untreated samples.

DISCUSSION

The electrophoretic mobility–pH curves for both cytochromoid *c* and cytochrome 552 exhibit some degree of similarity to each other and to that for ox-heart cytochrome *c*. It is noteworthy that the isoelectric points of the last two named are so close despite a disparity in the ratios of their basic to acidic amino acids. It is probable that this disparity is offset by the relatively higher ammonia content of cytochrome 552. Although for reliable ammonia determinations a series of hydrolyses under varying conditions would be required, the results indicate that most of the carboxylic acid side chains of cytochrome 552 are present as amides. Under these conditions the ε-amino group of lysine (p*K* 10·5) probably determines the net charge on the molecule at alkaline pH values. These results disagree with the isoelectric point of pH 7·7 reported by Morita (1960). Mobilities were obtained by him at only three pH values, namely 7·0, 7·5 and 8·0, so that only one determination was carried out on the alkaline side of the reported isoelectric point. Neither the actual mobility values obtained, nor the strain of *Rps. palustris* used, was given. In the absence of further evidence it can only be assumed that Morita (1960) obtained a cytochrome 552 having different properties from the above, perhaps from a mutant strain.

The lower electrophoretic mobilities of cytochromoid *c* compared with those of cytochrome 552 and ox-heart cytochrome *c* at about pH 7 may be explained in part by the relatively low ammonia content of the cytochromoid *c*. When the basic and acidic amino acid composition of this pigment is taken into account, together with its ammonia content, only a small net positive charge is indicated in this pH region. If the charge under these conditions is such that protein–ion interaction or aggregation of the cytochromoid *c* occurs, then this may explain the variable mobility values observed at near-neutral pH values (see e.g. Longsworth, 1959). It was found during starch-gel electrophoresis that oxidized cytochromoid *c* migrated more rapidly than the reduced form. A difference in migration rate during zone electrophoresis, dependent to some extent on the state of oxidation of the pigment, has long been known for heart-muscle cytochrome *c* (Paléus, 1952). It is now well

established that the latter pigment may, according to its method of preparation, be present in solution in several reversibly interchangeable polymeric forms (Margoliash & Lustgarten, 1962). It seems likely therefore that the variations in mobilities observed during starch-gel electrophoresis, which were dependent on the redox state of the pigments, were due to the presence of polymers.

Our preparations of cytochromoid *c* were initially autoxidizable and became significantly less so on treatment with alkali. This lends support to the view that, at least for the pigment from the above strain, it is the unmodified form that is autoxidizable.

We thank Dr C. A. M. Mauritzen for the amino acid analyses, Mr M. A. Marginson for an ammonia determination and Mr E. A. F. Matthaei for the photographic work.

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Biosynthesis of Nucleic Acid Purines in *Mycobacterium tuberculosis* $H_{37}R_v$

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(*Received* 26 *July* 1965)

1. The biosynthesis of nucleic acid purine in *Mycobacterium tuberculosis* $H_{37}R_v$ has been studied by using ^{14}C-labelled precursors. 2. The results indicate that C-2 and C-8 of the purine ring are derived most efficiently from serine and glycine and not from formate. 3. [^{14}C]Methionine is not incorporated into the ureide carbon atoms of the purine ring.

The investigations of Sonne, Buchanan & Delluva (1946*a*,*b*, 1948*a*,*b*) on the biosynthesis of purines in the avian system have revealed that C-2 and C-8 of the purine ring originate from formate. Further studies have shown that in mammalian and avian systems these carbon atoms can be derived not only from formate and formaldehyde, but also from the α-carbon of glycine and β-carbon of serine (Reichard, 1955; Schulman, 1961; Buchanan, 1960). But, in contrast with the mammalian and avian systems in which C_1 units from any precursor are incorporated equally to positions 2 and 8 of the purine ring, micro-organisms have been shown to exhibit certain differences in the precursors of these carbon atoms. Thus, growing cells of *Pseudomonas* and *Escherichia coli* can incorporate [^{14}C]formate into position 8 of the purine ring, but not appreciably into position 2 (Revel & Magasanik, 1958; Koch & Levy, 1955). Nester & Spizizen (1961) have also shown that in growing cells of *E. coli* formate is incorporated only into position 8 whereas isotope from [2-^{14}C]glycine and [3-^{14}C]serine is incorporated equally into positions 2 and 8 of the purine ring. Revel & Magasanik (1958) have shown that *Pseudomonas fluorescens* can utilize C-2 of histidine for the synthesis of C-8 of the purine ring. In micro-organisms possessing an active formate hydrogen-lyase, formate is rapidly oxidized to carbon dioxide, and in such systems other C_1 donors like serine and methionine may represent more efficient C_1 precursors than formate itself (Moat & Friedman, 1960). But few, if any, direct references to the methyl carbon of methionine as C_1 donor are available, even though several indirect implications may be found (Moat & Friedman, 1960). *Mycobacterium tuberculosis* is reported to oxidize formate (Franke & Schillinger, 1944) and hence the precursors of C-2 and C-8 of the nucleic acid purines in this organism have been the subject of the present investigation.

MATERIALS AND METHODS

Radioactive chemicals. [2-^{14}C]Glycine (8·1mc/m-mole), L-[3-^{14}C]serine (4·7mc/m-mole) and L-[*Me*-^{14}C]methionine (6·6mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. Sodium [^{14}C]formate (0·97mc/m-mole) was the product of the Atomic Energy Establishment, Trombay, India.

All other chemicals used were of the reagent grade.

Enzyme preparations. Xanthine oxidase was prepared from cream according to the method of Klenow & Emberland (1955). The second ammonium sulphate fraction (brown precipitate) was used as such.

Analytical methods. Purines were separated from extraneous materials by using ion-exchange column chromatography (Tyner, Heidelberger & LePage, 1952) and from each other by paper chromatography (Giri & Rao, 1952), identified and assayed spectrophotometrically (Wyatt, 1955). Urea was estimated by the colorimetric method of Barker (1944). All radioactive samples were plated on aluminium planchets and counted in a thin window Geiger–Müller counter. Since the amounts spotted were less than 0·03mg. no self-absorption correction was necessary.

Culture. The human virulent strain $H_{37}R_v$ of *M. tuberculosis* was the organism used in the present studies. The bacteria were grown at 37° on Youman's medium (Youman & Karlson, 1947) with glycerol as carbon source and asparagine as the nitrogen source. The conditions of growth were the same as described by Malathi, Sirsi, Ramakrishnan & Maller (1964).

Isolation of nucleic acid purines. The cells were collected by filtration on Whatman no. 1 filter paper and processed by the method of Marshak & Vogel (1951). The nucleic acids were extracted from the ice-cold 7% (w/v) trichloroacetic acid residue by the method of Schneider (1945). A portion (10mg.) of the residue was heated for 15min. at 90° with 1·5ml. of 5% trichloroacetic acid, cooled and centrifuged. The residue was resuspended in 0·5ml. of 5% trichloroacetic acid and centrifuged. The supernatants from both the centrifugations were pooled together and heated in a boiling-water bath for 2hr. to decompose the trichloroacetic acid (Hitchings, 1941). The solution was evaporated to dryness under reduced pressure and the

nucleic acids were hydrolysed with 1·0ml. of N-HCl at 100° for 1hr. The HCl was removed under reduced pressure and the purines were separated first by ion-exchange chromatography on a Dowex 50 (X8; H^+ form) column (1cm.× 2·5cm.) and further purified by circular paper chromatography on Whatman no. 3 filter paper with Kirby's (1955) solvent system methanol–ethanol–11·6N-HCl–water (50:25:6:19, by vol.). The purines were located by means of an ultraviolet lamp (Mineralight, SL2537). These areas, along with the corresponding bands in a blank area, were cut out and eluted with 4ml. of 0·1N-HCl and the paper was washed three times with 1·5ml. portions of the same acid. The pooled extracts were made up to 10ml. with acid and the concentration and radioactivity were determined as described above in Analytical methods; the specific activities of adenine and guanine were calculated.

Degradation of purines. The guanine obtained was evaporated to dryness under reduced pressure. It was taken up in 0·25ml. of 2·5N-H_2SO_4 and deaminated to xanthine by keeping it in a boiling-water bath and by dropwise addition of 0·2ml. of sodium nitrite (15mg./ml.). About 60–65% of the guanine was converted into xanthine. To this xanthine was added 10mg. of carrier xanthine dissolved in 0·5ml. of N-NaOH. The xanthine was precipitated twice by addition of N-H_2SO_4 with constant stirring. After washing thoroughly with water and ethanol it was dried over P_2O_5. About 2mg. was taken to determine the radioactivity. The rest was converted into alloxan and urea, which contains the C-8 moiety of the purine ring, by treatment with $KClO_3$ (Elwyn & Sprinson, 1954). Several drops of M-sodium thiosulphate were added to reduce any excess of chlorate. This urea–alloxan mixture was separated on a Dowex 50 (H^+ form) column (0·6cm.× 10cm.) and eluted with water (Nester & Spizizen, 1961). The first five fractions (4ml. each) contained alloxan; fractions 15–17 contained urea. The urea fractions were pooled and concentrated. Portions were taken for the determination of concentration and radioactivity. The alloxan was further degraded to urea containing C-2 of the purine ring and the urea separated on the Dowex 50 (H^+ form) column (Nester & Spizizen, 1961). The specific activities of C-8 and C-2 moieties of the guanine were calculated, based on the radioactive and colorimetric assays of the urea resulting from the degradation.

Adenine was deaminated, exactly as described for guanine, to hypoxanthine, which was converted into uric acid by the action of xanthine oxidase at pH7·4 and room temperature (Kalckar, 1947). To the reaction mixture, which had been freed from protein by heating in a boiling-water bath for 5min. and centrifugation, was added 10mg. of carrier uric acid in 0·5ml. of N-NaOH. The uric acid was twice precipitated from alkaline solution by the addition of N-H_2SO_4. The crystalline material was washed with water and ethanol and dried over P_2O_5. A small amount of uric acid was dissolved in N-NaOH. A portion of this was diluted with water and the concentration of uric acid

Table 1. *Incorporation of sodium* [^{14}C]*formate into the nucleic acid purines of* Mycobacterium tuberculosis *at different periods of growth*

The bacteria were grown in 75ml. of medium in the presence of different amounts (20·6μmoles and 41·2μmoles) of sodium [^{14}C]formate (310500counts/min./μmole). They were harvested at 8, 12 and 16 days. Other details are given in the text.

Growth period (days)	Wet wt. of cells (g.)	[^{14}C]Formate (counts/min./μmole)		Wet. wt. of cells (g.)	[^{14}C]Formate (counts/min./μmole)	
		Adenine	Guanine		Adenine	Guanine
8	0·150	540	490	0·210	865	840
12	0·730	215	191	0·620	404	323
16	1·070	132	137	0·780	309	223

Table 2. *Incorporation of various* C_1 *precursors into the nucleic acid purines of* Mycobacterium tuberculosis

The bacteria were grown in 37·5ml. of medium in the presence of various ^{14}C-labelled compounds in the following amounts: sodium [^{14}C]formate, 20·6μmoles (310500counts/min./μmole); [*Me*-^{14}C]methionine, 3·03μmoles (2112000counts/min./μmole); [2-^{14}C]glycine, 2·45μmoles (2592000 counts/min./μmole); [3-^{14}C]-serine, 4·25μmoles (1504000counts/min./μmole). Samples were taken at 8 and 16 days of growth. Details of the procedure are given in the text.

^{14}C-labelled supplement	Wet wt. of cells (g.)	8-day-old cells (counts/min./μmole)		Wet wt. of cells (g.)	16-day-old cells (counts/min./μmole)	
		Adenine	Guanine		Adenine	Guanine
Sodium[^{14}C]formate	0·175	1805	1417	0·600	357	539
[*Me*-^{14}C]Methionine	0·150	377	458	0·550	0	0
[2-^{14}C]Glycine	0·165	84700	79320	0·600	25630	25890
[3-^{14}C]Serine	0·200	73450	63400	0·600	23720	19710

Table 3. *Utilization of glycine and serine for the synthesis of* C-2 *and* C-8 *of the nucleic acid purines of* Mycobacterium tuberculosis

Adenine and guanine, isolated from the 8-day-old cells (Table 2) grown in the presence of [2-^{14}C]glycine and [3-^{14}C]serine, were used for degradation. Specific activities are given in Table 2. Other details are given in the text.

^{14}C-labelled supplement	Wet wt. of cells (g.)	Adenine (counts/min./μmole)		Guanine (counts/min./μmole)	
		C-8	C-2	C-8	C-2
[2-^{14}C]Glycine	0·165	18	18	50	58
[3-^{14}C]Serine	0·200	79	68	156	171

determined from the extinction of the solution at 290 mμ. Another portion of the same solution was taken for determination of radioactivity and the specific activity of uric acid calculated. Finally the uric acid was degraded in the same manner as xanthine.

RESULTS

The initial experiments designed to determine the efficiency of the incorporation of formate revealed that formate is incorporated only to a very small extent by 1-week-old cells and the specific activities of the purines decreased with the age of the culture (Table 1). Hence the experiment was repeated with other C_1 precursors. Table 2 summarizes the data on the incorporation of four C_1 precursors: [^{14}C]formate, [*Me*-^{14}C]methionine, [2-^{14}C]glycine and [3-^{14}C]serine. The results indicate that the specific activities were higher with 1-week-old cells than with the 2-week-old cultures.

It is also clear that *M. tuberculosis* is able to incorporate [2-^{14}C]glycine and [3-^{14}C]serine to a very significant extent into the nucleic acid purines. On the other hand, formate seems to be a very poor precursor and methionine proved to be still less efficient as a purine precursor. Degradation studies were conducted with the adenine and guanine samples obtained from 1-week-old cells grown in the presence of glycine and serine to get a precise knowledge of the extent of incorporation into C-2 and C-8 of the purine molecule. The results obtained are presented in Table 3. It is clear that the isotope from both the precursors is incorporated almost equally into both C-2 and C-8 of the purine ring. Since the incorporation with serine is about five times as great as that with glycine it is evident that serine is the more efficient precursor of the ureide carbon atoms of the purine ring in this organism. The possibility of the participation of methionine as C_1 donor in purine biosynthesis can be ruled out since its incorporation is insignificant.

DISCUSSION

The present studies have revealed that *M. tuberculosis* is capable of new biosynthesis of nucleic acid purines from simple precursors like glycine and serine. Formate, the classical precursor of the ureide carbon atoms of the purine ring, is not an efficient C_1 donor in this organism. Instead, both the ureide carbon atoms are derived most effectively from the β-carbon of serine and α-carbon of glycine. However, both the isotopes are incorporated almost equally into the C-2 and C-8 of the purine molecule.

Differential incorporation of the various precursors in micro-organisms has been observed by several other workers. Koch (1955) has shown that in growing cells of *E. coli* [2-^{14}C]glycine at a concentration of 0·1 μmole/ml. labelled position 8 exclusively whereas at higher concentrations (10 μmoles/ml.) the isotope contributed equally to positions 2 and 8. In contrast with this, Nester & Spizizen (1961) have observed that in *E. coli* both glycine (1 μmole/ml.) and serine (0·25 μmole/ml.) are incorporated equally into C-2 and C-8 of the purine ring. Again, Revel & Magasanik (1958) have shown that in *P. fluorescens* and *Aerobacter aerogenes* glycine even at low concentrations (0·5 μmole/ml.) contributes equally to C-2 and C-8. In the present investigation it is observed that in *M. tuberculosis* glycine (0·066 μmole/ml.) and serine (0·12 μmole/ml.), at concentrations even lower than those used by other workers, are incorporated almost equally into both C-2 and C-8.

Formate has been shown to be incorporated only into position 8 of the purine ring in several instances. Revel & Magasanik (1958) have shown that in *P. fluorescens* formate at low concentrations (0·5 μmole/ml.) is oxidized to carbon dioxide and therefore serves more efficiently as a donor of C-6 than of C-8, whereas at higher concentration of 20 μmoles/ml. it competes with histidine as a precursor of C-8 of the purine ring. In *A. aerogenes* formate has been shown to be incorporated only into C-8 of the purine ring (Revel & Magasanik, 1958). *E. coli* and other micro-organisms possess the enzyme formate hydrogen-lyase, and thus formate is rapidly converted into carbon dioxide, a considerable portion of which can be recovered from C-6 of the purines (Moat & Friedman, 1960). Nester & Spizizen (1961)

have shown that in *E. coli* formate is incorporated mostly into C-8 of the purine ring. In the present investigations, it has been found that formate even at the same concentration as that used in *E. coli* is incorporated into nucleic acid purines in *M. tuberculosis* only to the extent of 0·9% of that reported in *E. coli*. *M. tuberculosis* has been reported to oxidize formate (Franke & Schillinger, 1944), and thus formate may not be available as the C_1 donor. However, the extremely small amount of radioactivity may be present in C-6 or C-8 of the purine ring. No attempt has been made to study this since it was considered that the radioactivity in the individual carbon atoms on degradation would be too low to work with.

In systems that do not degrade formate the conversion of free formate into formyltetrahydrofolic acid represents the most efficient system for the C_1 transfer. But in *M. tuberculosis* serine and glycine represent the most effective C_1 donors which may contribute to the formation of formyltetrahydrofolic acid without going through a free formate stage. The participation of methionine as a C_1 donor has been discussed by Moat & Friedman (1960). Even though no direct evidence is available, several indirect implications may be found. Gots & Chu (1952) have found that a *p*-aminobenzoic acid-requiring mutant of *E. coli* accumulated 5-aminoimidazole-4-carboxamide under the conditions of *p*-aminobenzoic acid deficiency, or in the presence of competitive inhibitors of *p*-aminobenzoic acid, and this accumulation is depressed by methionine, suggesting that methionine may serve as donor of C-2. However, more detailed studies by Gots & Love (1954) have shown that this depression may be more likely due to a direct inhibitory effect on the new synthesis of 5-aminoimidazole-4-carboxamide and the contribution to C-2 could be unlikely because the organism was genetically deficient in transformylase. The present studies have revealed the inertness of methionine as the precursor of the ureide carbon atoms of the purine molecule in *M. tuberculosis*.

The results presented in this paper thus show that the biosynthesis of nucleic acid purines by *M. tuberculosis* differs in some essential respects from that by mammalian tissues.

The authors thank Dr M. Sirsi for his keen interest in the work and the Council of Scientific and Industrial Research, India, for a grant to carry out the investigation.

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Biochem. J. (1966) **98**, 598

Fatty Acid Composition of the Plasma Lipids of Newborn and Maternal Ruminants

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(*Received* 12 *August* 1965)

1. The fatty acid compositions of the plasma lipids of newborn unsuckled lambs, kids, calves and piglets have been determined and compared with those of maternal plasma lipids at parturition. 2. The predominating plasma fatty acids in the newborn of all species are palmitic acid, $C_{16:1}$ acid, stearic acid and $C_{18:1}$ acid. This finding is consistent with the synthesis of the major proportion of fatty acids from non-lipid sources within the foetus. 3. Very small amounts of $C_{18:2}$ acid and $C_{18:3}$ acid are present in the plasma lipids of newborn ruminants, although considerable amounts of these fatty acids are contained in maternal plasma. The plasma fatty acids of the newborn piglet contained 5·5% of $C_{18:2}$ acid, those of the calf 2·0%, and those of the lamb and kid less than 1·0%. This finding is discussed in relation to the higher content of $C_{18:2}$ acid in the plasma non-esterified fatty acid fraction of the sow (15%) compared with that of the ruminant (less than 4%). 4. In the lamb and kid, but not in the calf or piglet, a $C_{20:3}$ acid was detected in plasma lipids that was very similar to, if not identical with, the $C_{20:3}$ acid that accumulates in the plasma of animals given diets low in essential fatty acids. The possible significance of this finding is discussed. 5. The cholesteryl esters of cow plasma were found to contain a higher percentage (43%) of $C_{18:3}$ acid than those of goat and sheep plasma (5–10%). The possible reasons for this difference are discussed.

During an investigation of the plasma lipids of the neonatal lamb, it was noted that the total fatty acids of the plasma of newborn unsuckled lambs contained negligible amounts of octadecadienoic ($C_{18:2}$) acid. As the plasma of newborn monogastric animals contains appreciable amounts of $C_{18:2}$ acid (Satomura & Söderhjelm, 1962), it was decided to investigate the plasma fatty acids of other newborn ruminants to determine whether or not they were similar to the lamb. In addition, the plasma fatty acids of the sow and newborn piglet were examined as representatives of a non-ruminant farm animal. A preliminary account has been published (Leat, 1964).

EXPERIMENTAL

Animals. Blood samples were obtained from newborn unsuckled lambs, kids, calves and piglets, and from their mothers immediately after parturition. Samples were usually obtained from the external saphenous vein of newborn ruminants, from the jugular vein in adult ruminants, from the anterior vena cava of the piglet and from the ear vein of the sow. Blood was collected into chilled tubes containing a dry mixture (3:2, w/w) of ammonium oxalate and potassium oxalate (20mg./10ml. of blood) and stored in crushed ice until centrifuged at 4° to obtain the plasma. Samples of heart and liver were obtained from a foetal lamb estimated to be within 14 days of birth.

Extraction of plasma lipids. Plasma (5ml.) was poured into 50ml. of methanol–chloroform (1:1, v/v) containing 10mg. of quinol, and allowed to stand for 1hr. with occasional shaking. After filtration, 10ml. of the filtrate was evaporated almost to dryness in a rotary evaporator under reduced pressure; the residue was saponified and the methyl esters of the fatty acids were prepared as described previously (Leat, 1963). The remainder of the filtrate was similarly reduced to a small volume; the individual plasma lipids were separated by thin-layer chromatography on silica gel G and the methyl esters of each fraction were prepared as described by Bowyer, Leat, Howard & Gresham (1963).

Heart and liver samples. A 0·5g. sample of tissue was cut into small pieces and digested by heating with 10ml. of methanolic N-KOH containing 20mg. of quinol to minimize oxidation. After refluxing for 1hr. the fatty acids were extracted and the methyl esters prepared as above.

Gas–liquid chromatography. The methyl esters were separated at 180° on a 4ft. column of 10% polyethylene glycol adipate on 100–120 mesh Gas-Chrom Z (Applied Science Laboratories Inc., Pennsylvania, U.S.A.). Peaks were identified and estimated as described previously (Leat, 1963).

RESULTS

Total fatty acids of plasma. Table 1 shows that the most notable difference between the fatty acid composition of the newborn of all species and their mothers was in the content of $C_{18:2}$ acid, which was very much lower in the offspring. Among the newborn animals, piglets had the highest concentrations of $C_{18:2}$ acid (5%) and lambs and kids the lowest concentrations (less than 1%). Octadecatrienoic ($C_{18:3}$) acid was virtually absent from the newborn of all species although present in the mother, this difference being particularly noticeable between the cow and her calf. Compared with the mother, the newborn had higher concentrations of palmitic acid, $C_{16:1}$ acid and $C_{18:1}$ acid. The $C_{20:4}$ acid content of both maternal and newborn plasma was low in all animals studied, with the exception of the piglet. In all adult and infant animals there were trace amounts of an eicosatrienoic ($C_{20:3}$) acid having a carbon number of 21·2 (Woodford & Van Gent, 1960). In the lamb and kid, another eicosatrienoic acid of carbon number 21·0 was also present.

Plasma phospholipids (*Table* 2). The differences in fatty acid composition of the total lipids of newborn and maternal plasma were reflected in the phospholipid fatty acids. A lower content of stearic acid in the phospholipids of the newborn compared with the maternal plasma also became apparent. The high content of $C_{20:4}$ acid in the piglet is of interest. Although the composition of the phospholipids of the newborn and maternal plasma differed considerably, the percentage of total unsaturated acids was rather constant in both mother and offspring at about 50%.

Plasma cholesteryl esters (*Table* 2). The small amount of $C_{18:2}$ acid present in the plasma of the newborn appeared to be mainly associated with the cholesteryl ester fraction, where the major unsaturated acids were $C_{18:1}$ acid and $C_{16:1}$ acid. In the maternal plasma cholesteryl esters, the major unsaturated fatty acid in the pig, sheep and goat was $C_{18:2}$ acid, whereas in the cow $C_{18:3}$ acid predominated. Although $C_{18:3}$ acid accounted for nearly half of the fatty acids in cow plasma cholesteryl esters, only trace amounts were present in those of the calf. The fatty acids of the newborn of all species were more saturated than those of the mother, containing more palmitic acid and stearic acid.

Plasma triglycerides (*Table* 2). Adult ruminant plasma triglycerides were more saturated than those of the sow, containing considerably less $C_{18:2}$ acid and more stearic acid. Newborn ruminant triglycerides were more unsaturated than maternal triglycerides, containing less stearic acid and $C_{18:2}$ acid and more $C_{18:1}$ acid.

Plasma non-esterified fatty acids (*Table* 2). The plasma non-esterified fatty acids of the sow were more saturated than the triglycerides. In all the ruminants, however, the reverse was the case, with the plasma triglycerides being more saturated than the non-esterified fatty acids. The $C_{18:2}$ acid content of the sow plasma non-esterified fatty acids was much higher than that of ruminant plasma non-esterified fatty acids (15% compared with less than 4%). Of note is the high saturation of piglet plasma non-esterified fatty acids.

Total fatty acids of foetal liver and heart (*Table* 3). The low concentrations of $C_{18:2}$ acid noted in the plasma fatty acids of the lamb were also apparent in the fatty acids of liver and heart, but, despite

Table 1. *Percentage composition of the total fatty acids of maternal and newborn blood plasma*

The carbon no. is as defined by Woodford & Van Gent (1960), and the fatty acid designation is given as $x:y$, where x is the no. of carbon atoms/mol. and y is the no. of double bonds/mol. Each result given is the average of those for two animals. Some minor fatty acids have been omitted for clarity.

Carbon no.	Fatty acid designation	Fatty acid composition (%, w/w)							
		Sow	Piglet	Cow	Calf	Goat	Kid	Sheep	Lamb
14·0	14:0	1·3	2·1	1·5	1·5	1·0	2·2	1·2	1·8
16·0	16:0	19·4	23·1	11·5	32·3	17·5	24·1	16·7	22·5
16·3	16:1	4·2	10·5	3·1	11·0	2·9	13·0	3·3	8·4
18·0	18:0	14·0	11·8	9·5	10·2	12·7	6·8	12·2	9·3
18·3	18:1	18·7	36·9	15·4	39·5	27·6	49·5	28·3	50·0
18·9	18:2	34·2	5·5	24·9	2·0	29·5	<0·5	26·1	0·9
19·6	18:3	1·3	—	30·3	<0·5	3·4	—	4·6	<0·5
21·0	20:3	—	—	—	—	—	1·4	—	2·0
21·2	20:3	<0·5	1·1	0·6	0·8	<0·5	<0·5	1·0	0·8
21·5	20:4	1·8	6·3	1·0	1·2	3·0	0·5	2·1	1·2
Total unsaturated acids		60·2	60·3	75·3	54·9	66·7	64·7	65·4	63·6

Table 2. *Percentage composition of the major fatty acids of the component lipids of maternal and newborn blood plasma*

The fatty acid designation is defined in Table 1. Each result given is the average of those for two animals. Some minor fatty acids have been omitted for clarity.

Fatty acid designation	Fatty acid composition (%, w/w)							
	Sow	Piglet	Cow	Calf	Goat	Kid	Sheep	Lamb
(*a*) Cholesteryl esters								
14:0	1·5	2·9	2·1	2·8	1·6	2·6	0·9	0·9
16:0	12·3	24·6	7·4	29·0	17·0	23·5	14·6	32·8
16:1	7·0	16·5	4·3	16·2	5·6	24·5	2·6	13·8
18:0	5·5	7·3	1·9	7·7	6·7	8·2	5·9	11·0
18:1	16·7	34·0	6·4	28·0	24·3	28·3	27·1	35·7
18:2	48·2	5·8	29·8	5·5	34·1	1·5	37·0	1·5
18:3	0·6	0·5	43·7	0·8	5·0	0·9	10·2	<0·5
20:3	—	—	—	—	—	—	—	2·5
20:3?	—	—	0·6	1·0	—	—	<0·5	—
20:4	1·5	2·5	1·1	2·4	1·1	<0·5	0·7	—
Total unsaturated acids	74·0	59·3	85·9	53·9	70·1	55·2	76·9	52·1
(*b*) Phospholipids								
14:0	<0·5	0·7	0·9	0·5	<0·5	<0·5	<0·5	1·0
16:0	18·6	23·7	20·5	30·5	21·0	36·0	23·9	35·8
16:1	1·1	4·0	1·7	4·3	0·9	9·6	<0·5	6·6
18:0	34·6	25·7	25·2	21·4	28·8	10·8	26·4	15·1
18:1	16·3	26·2	16·9	34·0	14·5	36·6	20·3	33·7
18:2	23·8	4·4	15·8	1·7	22·6	<0·5	19·5	0·6
18:3	<0·5	0·7	11·0	<0·5	1·6	—	3·0	<0·5
20:3	—	—	—	—	—	3·2	—	3·2
20:3?	<0·5	0·5	1·3	1·9	<0·5	<0·5	<0·5	<0·5
20:4	4·4	11·8	1·8	3·5	4·1	2·2	2·5	2·2
Total unsaturated acids	46·0	47·6	48·5	45·4	43·7	51·6	45·7	46·3
(*c*) Triglycerides								
14:0	1·5	3·0	2·2	2·2	1·9	1·0	<0·5	<0·5
16:0	28·8	30·4	34·4	43·3	29·1	26·7	29·7	37·0
16:1	4·7	11·9	13·3	13·3	6·3	8·4	3·2	7·5
18:0	5·5	9·7	17·9	6·9	18·7	8·2	25·5	9·5
18:1	36·8	38·5	24·2	30·2	34·5	51·2	33·9	45·0
18:2	21·0	2·2	1·5	0·9	4·2	0·7	4·8	0·5
18:3	0·5	<0·5	1·0	—	0·9	0·6	—	—
Total unsaturated acids	63·0	52·8	40·0	45·4	45·9	60·9	41·9	53·0
(*d*) Non-esterified fatty acids								
14:0	0·8	3·9	1·6	1·3	1·3	0·8	<0·5	<0·5
16:0	34·9	43·2	21·2	31·3	17·9	17·5	17·8	21·8
16:1	5·3	5·2	4·3	8·1	3·5	3·9	0·9	4·1
18:0	17·6	21·1	22·6	11·3	23·1	14·5	34·9	16·2
18:1	20·8	15·2	45·8	43·8	46·8	62·0	41·8	57·6
18:2	15·2	4·0	1·5	1·3	3·6	<0·5	3·0	<0·5
18:3	1·3	—	<0·5	0·7	0·7	—	0·5	—
Total unsaturated acids	42·6	24·4	52·0	52·9	54·6	65·9	46·2	61·7

this, appreciable amounts of $C_{20:4}$ acid were present. Both eicosatrienoic acids were present, the one of carbon number 21·0 predominating. Analyses of adult sheep liver and heart are included for comparison.

DISCUSSION

Although adult ruminant plasma contains large amounts of $C_{18:2}$ acid and, in the cow, considerable amounts of $C_{18:3}$ acid also, very small amounts of these acids are present in the plasma of the newborn ruminant. In the foetal lamb these low concentrations of $C_{18:2}$ acid and $C_{18:3}$ acid are not confined to the plasma but are also apparent in the total fatty acids of liver and heart. Body & Shorland (1964) have also shown that the foetal lamb has very little fat in its carcass and that this fat contains very little $C_{18:2}$ acid. The newborn piglet, on the other hand, contains much more $C_{18:2}$ acid in its plasma than does the newborn ruminant. The

Table 3. *Fatty acid compositions of the liver and heart of a foetal lamb (estimated to be 14 days from birth) compared with those of an adult sheep*

The fatty acid designation is defined in Table 1.

	Fatty acid composition (%, w/w)			
	Sheep		Foetal lamb	
Fatty acid designation	Liver	Heart	Liver	Heart
14:0	0·5	1·1	<0·5	<0·5
15:0	0·2	<0·5	<0·5	<0·5
16:0	15·2	16·3	30·8	24·1
16:1	2·0	2·7	6·0	5·3
17:0	1·9	0·8	<0·5	<0·5
17:1	—	—	<0·5	<0·5
18:0	27·4	19·1	19·8	12·0
18:1	25·7	36·7	26·0	48·5
18:2	11·9	18·2	0·8	2·0
18:3	1·1	1·8	—	—
20:0	0·5	—	—	—
20:3	—	—	1·4	2·0
20:3?	0·4	<0·5	<0·5	<0·5
20:4	3·9	3·0	5·9	5·9
Higher acids	8·8	0·6	9·3	—

piglet therefore resembles other non-ruminants, e.g. rats, rabbits, guinea pigs and humans, where it has been shown that $C_{18:2}$ acid passes the placental membrane into the foetus (Söderhjelm, 1953; Satomura & Söderhjelm, 1962; Hansen *et al.* 1964). At first sight therefore it might appear that, in contrast with the non-ruminant, the ruminant placenta is relatively impermeable to $C_{18:2}$ acid and $C_{18:3}$ acid; but there may be another explanation. It has been shown that in rats, rabbits and guinea pigs the placenta is relatively impermeable to plasma cholesterol, phospholipids and chylomicron triglycerides (Goldwater & Stetten, 1947; Popják, 1954; McBride & Korn, 1964). However, in the sheep and the rabbit, plasma non-esterified fatty acids can pass the placental barrier into the foetus (Van Duyne, Parker, Havel & Holm, 1960; Van Duyne & Havel, 1960). In the present experiment, although the plasma phospholipids and cholesteryl esters of the sow and the ruminant contained high concentrations of $C_{18:2}$ acid, there was a striking difference in the $C_{18:2}$ acid content of the non-esterified fatty acid fraction. Whereas sow plasma non-esterified fatty acids contained 15% of $C_{18:2}$ acid, that in ruminant non-esterified fatty acids ranged from 1·5 to 3·6% (Table 2 and Fig. 1). If, as seems likely, the only plasma lipid to pass the placental membrane to any appreciable extent is the non-esterified fatty acid fraction, it might be expected that less $C_{18:2}$ acid would pass into the ruminant foetus than into the non-ruminant foetus. The same argument would apply to $C_{18:3}$ acid in the cow and calf.

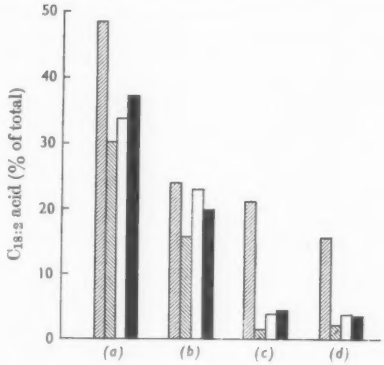

Fig. 1. Percentage of $C_{18:2}$ acid in the fatty acids of plasma (*a*) cholesteryl esters, (*b*) phospholipids, (*c*) triglycerides and (*d*) non-esterified fatty acids from the pig (■), cow (■), goat (□) and sheep (■).

This striking dissimilarity between the fatty acid composition of the plasma of the newborn lamb compared with its mother is at variance with the observations of Masters (1964), who showed that the fatty acid composition of the plasma of the newborn lamb resembled that of the adult sheep. However, in Masters's (1964) experiment 'the initial plasma samples were obtained a day or so after birth', during which time it is assumed that the lamb was allowed access to its mother's milk.

It is known that during the first 2 days of life considerable changes occur in the fatty acid composition of the plasma lipids of the lamb allowed to suck (Leat, 1964). At 48hr. after birth the fatty acid composition of lamb plasma is similar to that of the adult sheep, although completely different at birth.

In contrast with newborn ruminants, the plasma of the newborn piglet contains appreciable amounts of $C_{20:4}$ acid, which is associated mainly with the phospholipid fraction. In this respect the newborn piglet is similar to the newborn human infant, which has also been reported to contain large amounts of $C_{20:4}$ acid in its plasma (Hansen *et al.* 1964). The $C_{20:3}$ acid, noted in the plasma of lambs and kids, and in the heart and liver of the foetal lamb, has the same carbon number (21·0) as and similar properties to the fatty acid noted in the tissues of essential fatty acid-deficient rats and in pigs given diets low in linoleic acid (Leat, 1963). This fatty acid is probably the $\Delta^{5,8,11}$-eicosatrienoic acid characteristic of essential fatty acid deficiency (Mead, 1961). Holman (1960) has suggested that, in rats, a triene/tetraene ratio greater than 0·4 was indicative of a dietary insufficiency of essential fatty acids. If the same criterion is applicable to newborn lambs and to kids, it would appear that in these animals insufficient linoleic acid is passing the placental barrier to prevent the appearance of one of the characteristic metabolites in essential fatty acid deficiency in adult animals, i.e. eicosatrienoic acid. Whether or not the lamb and the kid can be said to be suffering from essential fatty acid deficiency is debatable, since information on the requirement of the young and mature ruminant is sparse. Further, the requirement of the foetus for linoleic acid is unknown and need not be the same as that of the adult animal. In the growing pig, for example, it has been shown that the requirement for linoleic acid is not constant but varies with age (Leat, 1962). When pigs are raised on low-fat diets varying degrees of centrilobular fatty changes occur in the liver (Gresham, Leat, Howard & Jennings, 1964). At this stage the plasma of these pigs contained about 1% of linoleic acid. Although lambs at birth have a similar concentration of plasma $C_{18:2}$ acid there is no evidence of any fatty change in the liver (W. M. F. Leat & T. Gillman, unpublished work).

In the calf it appears that the slightly higher concentration of $C_{18:2}$ acid in the plasma lipids is sufficient to prevent the appearance of the $C_{20:3}$ acid associated with essential fatty acid deficiency.

In samples of plasma lipids from all species examined here, small amounts of another fatty acid, having carbon number 21·2, were located between $C_{20:3}$ acid and $C_{20:4}$ acid on separation by gas–liquid chromatography. It is possible that this acid is the isomeric $\Delta^{8,11,14}$-eicosatrienoic acid, which is an intermediate in the formation of arachidonic acid from linoleic acid (Morin, Bernick, Mead & Alfin-Slater, 1962).

In all the newborn animals the major fatty acids of plasma were $C_{18:1}$ acid, palmitic acid, stearic acid and $C_{16:1}$ acid, which are acids that can be synthesized from non-fat sources. This agrees with the view that most of the foetal lipids are derived by synthesis within the foetus (Popják, 1954).

On fractionating the plasma lipids the differences in the fatty acid composition between newborn and maternal plasma became more apparent in some fractions than in others. Not obvious in the analysis of total fatty acids was the lower percentage of stearic acid in the phospholipids, triglycerides and non-esterified fatty acids of the plasma of newborn ruminants compared with their mothers. This lower percentage of stearic acid has also been noted in the glycerides of the organs and carcass of newborn and foetal lambs (Downing, 1964; Body & Shorland, 1964).

The cholesteryl esters of newborn ruminant plasma contained a higher percentage of $C_{18:2}$ acid than the other lipid fractions. The high concentration of $C_{16:1}$ acid is also of note since the acid is known to increase in amount in the cholesteryl esters of rats and pigs given insufficient linoleic acid (Howton & Hashimoto, 1960; Leat 1963). With the exception of the high concentration of $C_{18:3}$ acid in the cow, the fatty acid composition of the plasma cholesteryl esters and phospholipids of the sow and ruminants did not differ appreciably. The major difference between the ruminant and the non-ruminant lay in the fatty acid composition of the plasma triglycerides and non-esterified fatty acids.

The fatty acid composition of the plasma triglycerides of the cow, goat and sheep were similar and differed from that of the sow in being more saturated, containing less $C_{18:2}$ acid and more stearic acid. The high content of stearic acid in the plasma triglycerides of ruminants is probably a reflection of the large amounts of this acid produced by microbial action in the rumen. In agreement with previous work (Leat, 1963) the plasma non-esterified fatty acid fraction of the sow was found to be more saturated than the triglyceride fraction. In ruminants, however, the plasma non-esterified fatty acids were less saturated than the triglycerides, although containing more stearic acid. The high content of stearic acid in the plasma non-esterified fatty acids of ruminants might be expected since this fraction is derived mainly by mobilization from adipose tissue, which in ruminants has a high content of stearic acid (Hilditch, 1956).

The present results on the fatty acid composition of the plasma lipids of the sheep are in broad agree-

ment with previous work (Horgan & Masters, 1963; Garton & Duncan, 1964). The highest concentrations of $C_{18:2}$ acid and $C_{18:3}$ acid were in the cholesteryl esters, and the highest concentrations of stearic acid and palmitic acid were in the phospholipids and triglycerides. The fatty acid compositions of goat plasma lipids were very similar to those of the sheep. The $C_{18:1}$ acid contents of the cholesteryl esters and phospholipids of goat plasma reported in the present work are much lower than the values recorded by McCarthy (1962).

The fatty acids of cow plasma are characterized by the high content of $C_{18:3}$ acid, which is associated mainly with the cholesteryl esters and, to a smaller extent, with the phospholipids. These results are in good agreement with those reported by Duncan & Garton (1962), who suggested that the difference in the content of $C_{18:3}$ acid beween the cow and other ruminants might be related to diet or to the preferential esterification of $C_{18:3}$ acid with cholesterol in the cow. The cows studied in the present work had access to fresh grass, which is a rich source of linolenic acid, whereas the goats did not. However, the plasma cholesteryl esters of goats feeding on fresh pasture contained similar amounts of $C_{18:3}$ acid to those of goats that did not have access to fresh grass. In sheep feeding on fresh pasture the $C_{18:3}$ acid contents of plasma cholesteryl esters were somewhat higher than the values from stall-fed sheep. Also, the infusion of linseed oil, containing 67% of linolenic acid, directly into the duodenum of a sheep, thereby by-passing the hydrogenating mechanisms of the rumen, did increase the $C_{18:3}$ acid content of the plasma cholesteryl esters from 7 to 22% (W. M. F. Leat, unpublished work). However, this maximum value for sheep was only half the value noted in cows. It is therefore unlikely that possible differences between the cow and the sheep in the extent of microbial hydrogenation of linolenic acid in the rumen would entirely account for the considerable difference in the $C_{18:3}$ acid contents of their respective plasma cholesteryl esters. The results suggest that there is a greater tendency for $C_{18:3}$ acid to be esterified with cholesterol in the cow than in other ruminants.

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Ribonucleic Acid Synthesis during the Early Action of Thyroid Hormones

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(*Received* 26 *July* 1965)

1. The effect on RNA synthesis in rat liver of thyroidectomy and the administration of thyroid hormone, especially during its physiological latent period, was studied by determining: (*a*) the activity of DNA-dependent RNA polymerase in isolated nuclei; (*b*) the rate of synthesis of nuclear and cytoplasmic RNA *in vivo*; (*c*) polyribosomal sedimentation profiles; (*d*) the response of microsomes and ribonucleoprotein particles to polyuridylic acid; (*e*) the effect of inhibitors of RNA and protein synthesis on the biological activity of hormones. 2. The DNA-dependent RNA-polymerase activity of isolated rat-liver nuclei was lowered by thyroidectomy and stimulated by the administration of tri-iodo-L-thyronine or L-thyroxine (2–25μg./100g. body wt.) to both normal and thyroidectomized rats. In thyroidectomized rats, the activity of the Mg^{2+}-activated RNA-polymerase reaction (for which the product is mainly ribosomal type of RNA) was stimulated at 10–12hr. after a single injection of tri-iodothyronine, reaching a peak value of 60–90% stimulation at 45hr. after hormone administration. The Mn^{2+}/ammonium sulphate-activated RNA-polymerase reaction (for which the RNA product is more DNA-like) was not affected for 24hr. after hormone administration but stimulated by 30–40% at 45hr. The response of both RNA-polymerase reactions to the hormone *in vivo* paralleled the physiological response but the enzyme was not stimulated by the addition *in vitro* of the hormone to isolated nuclei. 3. Within 3–4hr. after tri-iodothyronine administration to thyroidectomized rats, the specific activity of rapidly labelled nuclear RNA, after a 10min. pulse of [6-^{14}C]-orotic acid, was 30–40% greater than the control values, the stimulation reaching 100 and 200% at 11 and 16hr. respectively after hormone administration. Longer exposures to [6-^{14}C]orotic acid and [^{32}P]phosphate showed that the hormone accelerated the synthesis of mitochondrial, microsomal (or ribosomal) and soluble RNA. The greater part of the labelled nuclear RNA was of the ribosomal type. The hormone-induced increases in the incorporation of radioactive precursors into RNA were not preceded, but followed, by enhanced uptake of the precursor. There was no change, per g. of liver, of DNA, nuclear RNA or soluble RNA, but there was a 40–60% increase in the amount of ribosomal RNA between 35 and 45hr. after a single injection of tri-iodothyronine to thyroidectomized rats. 4. Coinciding with the increase in ribosomal RNA after hormone administration was an increase in the average size and amount of polyribosomes. The newly formed ribonucleoprotein particles, or messenger RNA attached to them, or both, were more firmly bound to microsomal membranes after hormone treatment. 5. Polyuridylic acid caused a bigger stimulation of incorporation of [^{14}C]phenylalanine by ribonucleoprotein particles, but not by microsomes, from thyroidectomized rats as compared with preparations from normal animals. The response of ribonucleoprotein particles to polyuridylic acid was lowered after tri-iodothyronine treatment of thyroidectomized rats. 6. Actinomycin D, 5-fluorouracil, puromycin and cycloheximide caused a 70–100% inhibition of the stimulatory effect of L-thyroxine and tri-iodo-L-thyronine on basal metabolic rate and growth rate in both normal and thyroidectomized animals. Administration of actinomycin D also abolished the stimulation of RNA polymerase by tri-iodothyronine. 7. It is concluded that regulation of nuclear and ribosomal RNA synthesis is an essential step leading to the biological action of thyroid hormones and that the formation of new ribosomes is an important aspect of the control of cytoplasmic protein synthesis by these hormones.

A single injection of small amounts of thyroid hormone causes, after a latent period of about 30hr., a stimulation of the incorporation of amino acids into protein by microsomes and mitochondria (Tata *et al.* 1963; Roodyn, Freeman & Tata, 1965). Regulation of cytoplasmic protein synthesis is thought to be the basis for the growth-promoting and metabolic actions of L-thyroxine and 3,3′,5-tri-iodo-L-thyronine (see Tata, 1964*a*). The importance of all types of RNA in the control of protein synthesis, the observation that the stimulatory effects of thyroid hormones *in vivo* were accompanied by changes in the cytoplasmic RNA concentrations and the latent period of nearly 30hr. necessary for this effect to occur (Tata *et al.* 1963; Roodyn *et al.* 1965) prompted us to examine the possible influence these hormones may exert on the ability of the cell nucleus to synthesize RNA. At the time we initiated the studies reported below, there was no information on the effects of thyroid hormones on nucleic acid synthesis. Since then several reports have emphasized the importance of RNA synthesis in the action of other growth-promoting and developmental hormones (Korner, 1964; Williams-Ashman, Liao, Hancock, Jurkowitz & Silverman, 1964; Mueller, 1965; Sekeris, 1965).

We describe in the present paper the regulation of RNA synthesis by thyroid hormones, especially during the early period of their physiological action. This has been investigated from four different angles: (*a*) the activity of DNA-dependent RNA polymerase (EC 2.7.7.6) in isolated nuclei after thyroidectomy has been followed as a function of time after thyroid hormone administration; (*b*) the rate of incorporation of radioactive precursors into nuclear and cytoplasmic RNA *in vivo* has been followed under the same conditions; (*c*) polyribosomal sedimentation profiles and the response of microsomes and ribosomes to synthetic messenger RNA that accompany the changes in cytoplasmic protein synthesis induced by the hormone *in vivo* have been determined; (*d*) the effect of inhibitors of RNA and protein synthesis on the two major biological actions of thyroid hormones in mammals, namely growth promotion and regulation of B.M.R.,* were measured. We conclude from these studies that the regulation of RNA synthesis in the nucleus and the formation of new ribosomes are important features of the early action of thyroid hormones. Some of the results have already been reported briefly (Widnell & Tata, 1963; Tata, 1963, 1964*b*).

* Abbreviations: B.M.R., basal metabolic rate; s-RNA, 'soluble' RNA; m-RNA, messenger RNA; RNP, ribonucleoprotein; polyU, polyuridylic acid.

METHODS AND MATERIALS

Animals and treatment. Male albino rats (Wistar) were used, with no restrictions on their food intake throughout the experiments. Thyroidectomy was performed on young animals (45–55g.) by a combination of surgery and radioiodine administration. Thyroidectomized rats were used about 4 weeks after the operation by which time their B.M.R. and body weight were about 20 and 30% respectively below the values in unoperated controls. Periodic checks were made on B.M.R. and growth rate as described by Tata *et al.* (1963). The usual dose of 3,3′,5-tri-iodo-L-thyronine administered as a single subcutaneous injection was about 20μg./100g. body wt. Radioactive substances and inhibitors of RNA and protein synthesis were dissolved in 0·15M-NaCl and injected intraperitoneally.

Isolation of subcellular fractions. All procedures were performed at 0–4°. For studies on isolated nuclei and RNA synthesis *in vivo*, livers from three rats were pooled and homogenized in 0·32M-sucrose containing $MgCl_2$ (3mM). Nuclei were isolated as described by Widnell & Tata (1964*a*) and 55–70% of the total DNA in the homogenate was recovered in the nuclear fraction. Mitochondria, microsomes (or ribosomes) and the cell sap were separated by differential centrifuging (Tata *et al.* 1963) from the nuclei-free homogenate after a preliminary additional centrifuging at 700*g* for 10min. For studies on the incorporation of amino acids into protein and distribution of polyribosomes, microsomes and ribosomes were isolated from livers homogenized in a medium containing sucrose (0·25M), KCl (75mM), $MgCl_2$ (10mM) and tris–HCl buffer, pH7·6 (35mM), as described by Tata *et al.* (1963).

Distribution of polyribosomes. Ribosomes from 2g. of liver were obtained from the mitochondria-free supernatant by treatment with 0·25–1·0% (w/v) sodium deoxycholate and were suspended in 1ml. of a medium containing KCl (50mM), $MgCl_2$ (1mM) and tris–HCl buffer, pH7·6 (20mM). These were then layered over 26ml. of 15–30% (w/v) discontinuous sucrose density gradient (Charlwood, 1963) with a cushion of 3ml. of 60% (w/v) sucrose at the bottom of the Spinco SW25.1 rotor tube. After centrifuging at 22000 rev./min. for 110–140min. at 0–4°, 1ml. fractions were removed by siphoning from the bottom of the tube and extinctions at 260mμ and 280mμ and radioactivity were determined in the fractions (E_{260} 1·0 corresponds to 50μg. of RNA). The pellet that had sedimented through the sucrose gradient was suspended in 3ml. of 1·0% sodium deoxycholate and corrections were made for protein content from the E_{260}/E_{280} ratio. The RNA, in the remainder of each fraction after samples had been removed for extinction measurement, was precipitated with 0·4N-$HClO_4$, and washed with 70% (v/v) ethanol before ^{14}C determination.

Assay of RNA polymerase. The two RNA-polymerase reactions of isolated rat-liver nuclei were determined by a slight modification of the procedure described by Widnell & Tata (1964*b*). The incubation medium for the Mg^{2+}-activated reaction, carried out in the absence of $(NH_4)_2SO_4$, contained, in 0·5ml.: 50μmoles of tris–HCl buffer, pH8·5; 2·5μmoles of $MgCl_2$; 10μmoles of cysteine; 3μmoles of NaF; 0·3μmole each GTP, CTP and UTP; 0·015μmole of non-radioactive ATP; 0·005μmole of [^{14}C]ATP; 0·1ml. of nuclear suspension containing about 0·3mg. of DNA. Incubation was for 15min. in air at 37°, after which time the reaction was stopped by the addition of 5ml. of 0·5N-$HClO_4$ at 0°.

The incubation medium for the Mn^{2+}-activated RNA-polymerase reaction, carried out in the presence of $(NH_4)_2SO_4$, contained, in 0·5ml.: 50μmoles of tris–HCl buffer, pH 7·5; 2μmoles of $MnCl_2$; 0·05ml. of a solution of $(NH_4)_2SO_4$, saturated at room temperature and adjusted to pH 7·5 with aq. NH_3; 0·3μmole each of GTP, CTP and UTP; 0·015μmole of non-radioactive ATP; 0·1ml. of nuclear suspension. After preincubation for 15min. at 37°, the reaction rate was followed by the addition of 0·005μmole of [^{14}C]ATP, and the reaction was stopped, after a further 45min. of incubation, by the addition of 5ml. of 0·5N-$HClO_4$ at 0°.

The RNA product of both reactions was extracted, and the radioactivity determined, as described by Widnell & Tata (1964*a*). Unless otherwise stated, each value presented for the specific activity of RNA polymerase represents the mean of either triplicate determinations at one concentration of nuclei in the incubation medium, or duplicate determinations at two concentrations; these values normally agreed to within 5%.

Other enzymic determinations. NAD pyrophosphorylase was determined as described by Widnell & Tata (1964*a*). ATP was determined enzymically by the method of Roodyn & Suttie (1961).

Nuclear RNA and cytoplasmic RNA labelled in vivo. Rats were killed at different time-intervals after the injection of 4–8μc of [^{14}C]orotic acid or 60–80μc of carrier-free [^{32}P]-orthophosphate and their livers were homogenized and subjected to subcellular fractionation. Nuclei, mitochondria and microsomes (or ribosomes) were suspended in an all-glass Potter–Elvehjem homogenizer as 2g. equivalent of liver/ml. of the initial homogenizing medium. With microsomes, sodium deoxycholate was added to the suspending medium at a final concentration of 0·4%. RNA from the 1·0–2·5ml. suspensions of the different particles and 12ml. of cell sap (105000*g* supernatant) was precipitated with 0·4N-$HClO_4$ at 0°. The precipitate was washed three times with 0·4N-$HClO_4$ and then twice with ether–ethanol (3:1, v/v) and once with ether alone before two extractions of the RNA with hot 2M-NaCl (90° for 60 and 30min. for the first and second extractions respectively). One sample of the hot-NaCl extract was used for chemical determination of RNA; 200μg. of carrier yeast RNA was added to the rest, and the RNA precipitated with 66% (v/v) ethanol and hydrolysed in 0·5ml. of 0·4N-KOH or -NaOH at 35° for 18hr. The hydrolysate was neutralized with $HClO_4$ or HCl, any precipitate removed by centrifuging and the DNA precipitated by the addition of 2vol. of ethanol. The ribonucleotides in the ethanol supernatant were concentrated for the determination of radioactivity and chromatography.

Incorporation of L-[^{14}C]*phenylalanine into protein and the effect of polyU.* The incubation mixture contained, in a final volume of 0·6ml.: 3μmoles of KCl; 14μmoles of tris–HCl buffer, pH 7·6; 4·2μmoles of $MgCl_2$; 0·09μmole of each of 19 amino acids except for phenylalanine; 6·0μmoles of GSH; 2·5μmoles of phosphoenolpyruvate; 0·25μmole of ATP; 0·0625μmole of GTP; 25μg. of pyruvate kinase; L-[^{14}C]phenylalanine. The amino acid mixture was made up of: L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glycine, L-glutamine, L-glutamic acid, L-histidine, L-hydroxyproline, L-isoleucine, DL-leucine, L-lysine, L-methionine, L-proline, L-serine, L-tryptophan, L-tyrosine and L-valine. The reaction was stopped by the addition of 5μmoles of non-radioactive phenylalanine in 0·2ml. of N-NaOH; the incubation was continued for another 5min. to hydrolyse the remaining s-RNA, after which protein was precipitated with 2·0ml. of 10% (w/v) trichloroacetic acid at 0°. The precipitate was washed three times with 3ml. of cold 5% (w/v) trichloroacetic acid, twice with the hot acid and twice with water on Oxoid membrane filters (Oxo Ltd., London, E.C.4). After drying, the radioactivity in the

Table 1. *Effect of thyroidectomy, and of thyroid hormone administration* in vivo *to thyroidectomized and normal rats, on the specific activities of RNA polymerase and NAD phosphorylase in isolated rat-liver nuclei*

Experimental details are given in the text. The specific activity of the Mg^{2+}-activated RNA-polymerase reaction is expressed as $\mu\mu$moles of [^{14}C]ATP incorporated into RNA/15min./mg. of DNA, and that of the $Mn^{2+}/(NH_4)_2SO_4$-activated RNA-polymerase reaction as $\mu\mu$moles of [^{14}C]ATP incorporated into RNA/45min./mg. of DNA. The specific activity of NAD pyrophosphorylase is expressed in mμmoles of NAD synthesized/20min./mg. of DNA.

Animals	Specific activity of RNA polymerase: Mg^{2+}-activated	Specific activity of RNA polymerase: $Mn^{2+}/(NH_4)_2SO_4$-activated	Specific activity of NAD pyrophosphorylase
Thyroidectomized	665	2205	492
Thyroidectomized, treated with 25μg. of tri-iodothyronine 24hr. before death	1076	2325	475
Thyroidectomized, treated with 25μg. of tri-iodothyronine 45hr. before death	1085	3225	508
Normal, unoperated	1135	3275	502
Normal unoperated, treated with 5μg. of tri-iodothyronine/day for 8 days before death	1445		

protein was directly determined on the filters. For comparing the response of microsomes and ribonucleoprotein particles to polyU, the usual time of incubation at 37° was 30 and 60 min. (Campbell, Cooper & Hicks, 1964).

Determination of nearest-neighbour frequencies and base composition of RNA. For the determination of nearest-neighbour frequencies of the four nucleotides of the hydrolysed product of RNA polymerase, the nucleotides were separated by the ion-exchange column-chromatographic method of Katz & Comb (1963) and specific radioactivity was measured in the eluates. For the determination of base composition of ^{32}P-labelled RNA formed *in vivo*, the nucleotides extracted into ethanol from the RNA hydrolysate were separated by paper chromatography by the method of Lane (1963). The relative distribution was estimated from the density of silver grains deposited on a radioautograph (Ilford, No-Screen X-ray film), by using a Joyce–Loebl double-beam densitometer.

Measurement of radioactivity. ^{14}C was generally measured in the Packard Tri-Carb scintillator spectrometer by depositing the RNA on a cylinder of filter paper (Widnell & Tata, 1964*a*) (counting efficiency 66%, background 20 counts/min.). The scintillator fluid contained 0·5% of 2,5-diphenyloxazole and 0·05% of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in redistilled toluene. In studies on the incorporation of [^{14}C]phenylalanine into protein, the radioactivity was measured on the membrane filter in a Nuclear–Chicago thin-window gas-flow counter (efficiency about 20%, background $2 \pm 0{\cdot}5$ counts/min.). ^{32}P was also measured with an end-window Geiger–Müller counter (efficiency about 40%).

Chemical determinations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as standard. DNA and RNA were estimated by the methods of Burton (1956) and Ceriotti (1955) respectively, after extraction of the nucleic acids as described by Widnell & Tata (1964*a*), with calf-thymus DNA (Sigma) and yeast RNA (Boehringer) as standards. Phosphorus was measured by the method of Fiske & Subbarow (1925).

Materials. ATP, GTP, CTP, UTP, NAD and NMN were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; yeast RNA, phosphoenolpyruvate, GSH, yeast alcohol dehydrogenase and pyruvate kinase were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; amino acids, nicotinamide and 5-fluorouracil were from Roche Products Ltd., Welwyn Garden City, Herts. Puromycin was obtained from Lederle Laboratories, Pearl River, N.Y., U.S.A., cycloheximide (Actidione) was from California Corp. for Biochemical Research, Lucerne, Switzerland, and actinomycin D was a gift from Merck, Sharpe and Dohme Co. Inc., Rahway, N.J., U.S.A. L-Thyroxine, 3,3′,5-tri-iodo-L-thyronine and their derivatives were obtained from Glaxo Laboratories, Greenford, Middlesex; they were dissolved in 1% (v/v) propane-1,2-diol at pH 8·3–8·5. PolyU was purchased from Miles Chemical Co., Elkhart, Ind., U.S.A., and dissolved in water.

Carrier-free [^{32}P]phosphate, L-[^{14}C]phenylalanine (1·22 mc/m-mole) and [6-^{14}C]orotic acid (6·8 mc/m-mole) were purchased from The Radiochemical Centre, Amersham, Bucks. [8-^{14}C]ATP (11·2 mc/m-mole) was obtained from Schwarz Bio-Research Inc., Orangeburg, N.Y., U.S.A. [α-^{32}P]ATP and [α-^{32}P]GTP were synthesized as described by Nakamoto, Fox & Weiss (1964).

RESULTS

Effect of thyroid hormone administration in vivo on RNA-polymerase activity in vitro. Preliminary experiments (Widnell & Tata, 1963) showed that the specific activity of the Mg^{2+}-activated RNA-polymerase reaction of isolated nuclei was stimulated within 16 hr. after the administration of tri-iodo-L-thyronine to thyroidectomized rats. In a more detailed investigation (Table 1), it was found that the specific activities of both the Mg^{2+}- and Mn^{2+}/ammonium sulphate-activated RNA-polymerase reactions were depressed by thyroidectomy, and restored to normal 45 hr. after a single administration of tri-iodo-L-thyronine to thyroidectomized

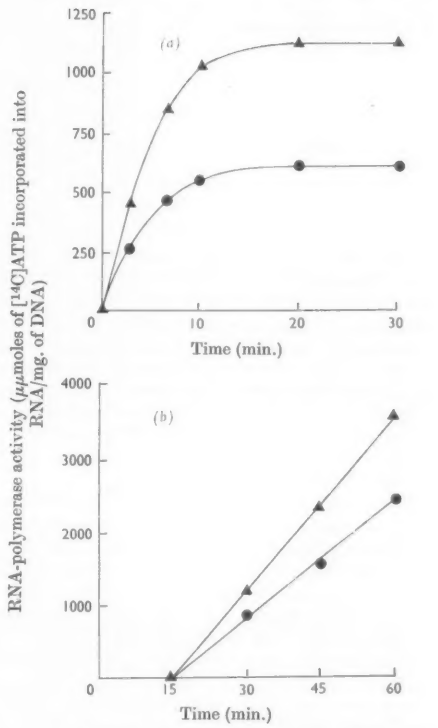

Fig. 1. Time-course of the two RNA-polymerase reactions in liver nuclei isolated from thyroidectomized and tri-iodothyronine-treated thyroidectomized rats. The assay of the two RNA-polymerase reactions was as described in the text. (*a*) Mg^{2+}-activated RNA-polymerase reaction; (*b*) $Mn^{2+}/(NH_4)_2SO_4$-activated RNA-polymerase reaction. ●, Nuclei isolated from thyroidectomized rats; ▲, nuclei isolated from thyroidectomized rats 45 hr. after an injection of 25 μg. of tri-iodothyronine/100 g. body wt.

Table 2. *Characteristics of the two RNA-polymerase reactions of liver nuclei isolated from thyroidectomized and tri-iodothyronine-treated thyroidectomized rats*

Experimental details are given in the text. The deoxyribonuclease treatment was as described by Widnell & Tata (1966*b*). Tri-iodothyronine-treated rats received a single injection of 20 μg. of the hormone 45 hr. before death.

Source of nuclei	Incubation medium	RNA-polymerase activity ($\mu\mu$moles of [^{14}C]ATP incorporated into RNA/mg. of DNA)	
		Mg^{2+}-activated	Mn^{2+}/$(NH_4)_2SO_4$-activated
Thyroidectomized rats	Complete	684	2320
	GTP omitted	193	274
	CTP omitted	184	301
	UTP omitted	167	359
	GTP, CTP and UTP omitted	61	117
	Complete, +25 μg. of deoxyribonuclease	109	42
Tri-iodothyronine-treated thyroidectomized rats	Complete	1240	3540
	GTP omitted	379	336
	CTP omitted	342	352
	UTP omitted	296	397
	GTP, CTP and UTP omitted	78	139
	Complete, +25 μg. of deoxyribonuclease	187	97

rats. Table 1 also shows that the specific activity of the Mg^{2+}-activated RNA-polymerase reaction was stimulated 24 hr. after tri-iodo-L-thyronine administration, at which time no change was detected in the specific activity of the Mn^{2+}/ammonium sulphate-activated RNA-polymerase reaction. The nuclei of normal rats also responded to the administration of thyroid hormone but to a smaller extent than preparations from thyroidectomized rats. No change was observed, in any of the groups of experimental animals, in the specific activity of NAD pyrophosphorylase.

The major characteristics of the two RNA-polymerase reactions were not altered by thyroidectomy or by the administration of tri-iodo-L-thyronine to thyroidectomized rats. Fig. 1 shows that the incubation time-course of both RNA-polymerase reactions was the same in liver nuclei isolated from either untreated or tri-iodothyronine-treated thyroidectomized rats. In the Mn^{2+}/ammonium sulphate-activated RNA-polymerase reaction, the radioactive precursor was added after 15 min. of incubation since by this time no more RNA was formed by the reaction taking place in the absence of the salt (see Widnell & Tata, 1964*b*). Table 2 shows that for both groups of animals, and for both RNA-polymerase reactions, incorporation of [^{14}C]ATP into RNA was dependent on DNA as well as on the presence of all four nucleoside triphosphates. The stimulation of RNA polymerase after tri-iodothyronine treatment could not be explained by a decrease in the size of the endogenous ATP pool of isolated nuclei, as the concentration of ATP in liver nuclei from thyroidectomized rats, which was found to be less than 1 mμmole/mg. of DNA, was not sufficiently large to exert any effect on the specific activity of the enzyme. From this and the lack of effect on nuclear NAD pyrophos-

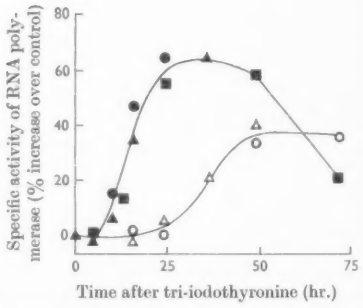

Fig. 2. Stimulation of the two RNA-polymerase reactions of isolated liver nuclei after a single injection of 25 μg. of tri-iodothyronine/100 g. body wt. to thyroidectomized rats. The experimental details were as described in the text. ●, ▲ and ■, Mg^{2+}-activated RNA-polymerase reaction; ○ and △, Mn^{2+}/$(NH_4)_2SO_4$-activated RNA-polymerase reaction. Different symbols denote the values obtained from separate experiments.

phorylase, it may be inferred that the effects of thyroidectomy and thyroid hormone replacement on the specific activity of RNA polymerase are unlikely to be due to some non-specific effect on nuclear metabolic activity, but that they represent a more selective action on RNA polymerase.

Analysis of the time-course of the hormonal stimulation revealed quite distinct responses of the two RNA-polymerase reactions to a single injection of tri-iodothyronine to thyroidectomized rats, as shown in Fig. 2. No change was detected in the specific activity of the Mg^{2+}-activated reaction 6hr. after the single injection, but at 10hr. a small but reproducible rise was observed. Peak stimulation was between 24 and 48hr., after which time the specific activity gradually fell, returning to the control value by 140hr. Essentially no change was observed in the specific activity of the Mn^{2+}/ammonium sulphate-activated reaction during the first 24hr. after a single injection of tri-iodothyronine, but by 42hr. the specific activity was stimulated to values found in nuclei isolated from normal animals; this stimulated level was maintained until at least 72hr. after the injection. No change was observed in the protein/DNA or RNA/DNA ratio of any of the nuclear preparations described in Fig. 2.

The pattern of the dose response of both RNA-polymerase reactions to a single injection of tri-iodo-L-thyronine, which is shown in Fig. 3, was very similar to that found for whole body growth or increase in the weight of the liver (J. R. Tata & C. C. Widnell, unpublished work). Maximum stimulation of either reaction to give values close to those of the specific activity in the control unoperated animal was observed at a dose of about 2μg. of tri-iodo-L-thyronine/100g. body wt. There was also a good correlation between the physiological activity of structural analogues of thyroid hormones in the whole animal (see Pitt-Rivers & Tata, 1959) and their effects on RNA polymerase determined in isolated nuclei. Table 3 shows that the analogues tri-iodo-D-thyronine and 3,5-di-iodo-L-thyronine, which possess only a fraction of the biological activity of tri-iodo-L-thyronine, caused a slight stimulation of both RNA-polymerase reactions, but that the stimulation at high doses

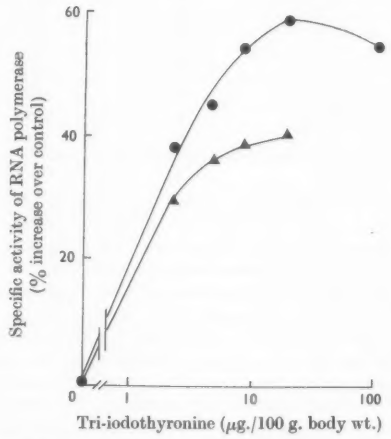

Fig. 3. Effect of the dose of tri-iodothyronine administered to thyroidectomized rats on the stimulation of the two RNA-polymerase reactions of isolated liver nuclei. The animals were killed 45hr. after hormone administration; other experimental details were as described in the text. ●, Mg^{2+}-activated RNA-polymerase reaction; ▲, Mn^{2+}/$(NH_4)_2SO_4$-activated RNA-polymerase reaction.

Table 3. *Stimulation of the two RNA-polymerase reactions of isolated liver nuclei by thyroid hormone analogues administered* in vivo

Experimental details for the assay of the two RNA-polymerase reactions are as given in the text. The specific activity of RNA polymerase is as expressed in Table 1. Compounds were injected as a single dose 45hr. before killing the animals.

Rats	Compound	Amount injected (μg.)	Specific activity of RNA polymerase: Mg^{2+}-activated	Specific activity of RNA polymerase: Mn^{2+}/$(NH_4)_2SO_4$-activated
Normal	—	—	960	3020
Thyroidectomized	—	—	704	2170
	3,3′,5-Tri-iodo-L-thyronine	18	1255	3080
	3,3′,5-Tri-iodo-L-thyronine	2	1105	2870
	3,3′,5-Tri-iodo-D-thyronine	30	1040	2750
	3,5-Di-iodo-L-thyronine	150	1010	2650
	L-Thyroxine	30	1180	Not assayed

Table 4. *Effect of tri-iodo-L-thyronine treatment* in vivo *and* in vitro *on the* Mg^{2+}*-activated RNA-polymerase reaction of liver nuclei isolated from thyroidectomized rats*

Experimental details are given in the text. The specific activity of the Mg^{2+}-activated RNA-polymerase reaction is as expressed in Table 1.

Time after tri-iodo-L-thyronine treatment *in vivo* (hr.)	Tri-iodo-L-thyronine added to nuclei *in vitro* (μg.)	Specific activity of RNA polymerase
—	—	626
—	0·1	618
—	1·0	628
—	10·0	594
16	—	838
48	—	964

of the analogues was lower than that induced by a small dose of both the normally circulating hormones, tri-iodo-L-thyronine and L-thyroxine.

Table 4 shows that, under conditions in which treatment of thyroidectomized rats with tri-iodo-L-thyronine *in vivo* caused a stimulation of the Mg^{2+}-activated RNA-polymerase reaction, direct addition *in vitro* of tri-iodothyronine to nuclei isolated from the livers of thyroidectomized rats had no effect on the specific activity. No effect was observed at any concentration of tri-iodothyronine from 0·1 μg. to 10 μg. added to 0·5 ml. of the incubation mixture. This result, taken together with the finding (Fig. 2) that no change could be detected in the specific activity of this RNA-polymerase reaction until about 10 hr. after tri-iodo-L-thyronine administration, suggests that the hormonal stimulation of RNA polymerase *in vivo* does not stem from a direct hormone–enzyme interaction.

Effect of thyroid hormone on the synthesis and turnover of nuclear RNA and cytoplasmic RNA in vivo. [6-^{14}C]Orotic acid was found to be more suitable than [^{32}P]phosphate as a precursor for studying the formation of rapidly labelled nuclear RNA in rat liver. Fig. 4 shows the time-course of incorporation of ^{14}C into RNA extracted from nuclei, mitochondria, microsomes and cell sap of livers of thyroidectomized rats after the injection of [^{14}C]orotic acid. This pattern is similar to that described for normal rat liver by other workers who have studied the incorporation of [^{14}C]orotic acid into RNA (Adams & Busch, 1963; Moulé & Delhumeau de Ongay, 1964). At early time-intervals after the administration of [^{14}C]orotic acid, over 75% of acid-insoluble ^{14}C was recovered as nuclear RNA, whereas at time-intervals exceeding 2 hr. an increasing fraction of radioactivity appeared in cytoplasmic RNA. This

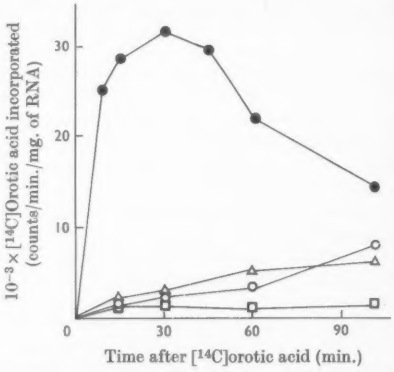

Fig. 4. Time-course of incorporation of [^{14}C]orotic acid into nuclear and cytoplasmic RNA from livers of thyroidectomized rats. A 5·0 μc dose of [^{14}C]orotic acid was injected into each rat at the various time-intervals indicated before killing and the RNA extracted from the different cellular fractions as described in the text. ●, Nuclear RNA; △, mitochondrial RNA; ○, microsomal RNA; □, s-RNA.

pattern is consistent with the conclusion drawn from kinetic analysis in various mammalian and microbial systems that cytoplasmic RNA is formed in the nucleus (see Prescott, 1964).

Administration of tri-iodothyronine to thyroidectomized rats caused an early enhancement of the incorporation of [^{14}C]orotic acid into the rapidly labelled nuclear RNA (Table 5). The specific radioactivity of nuclear RNA, after a 10 min. pulse of labelled orotic acid, was over 3 times the control value in animals that had received a single injection of tri-iodothyronine 16 hr. before. No further stimulation was discernible if the animals were pretreated with the hormone for longer periods of time. The earliest significant increase (25% over the control values) in the specific activity of the rapidly labelled nuclear RNA was observed 3–4 hr. after the hormone administration, with a doubling of the specific activity at about 12 hr. A marked elevation of the total uptake of ^{14}C from [^{14}C]orotic acid (acid-soluble and acid-insoluble ^{14}C) was observed 16 hr. after tri-iodothyronine but not at earlier time-intervals, suggesting that the higher uptake of the precursor may reflect an adjustment to an accelerated rate of RNA synthesis.

In experiments in which the incorporation of [^{32}P]phosphate into RNA was studied after longer exposure to the isotope than with orotic acid, a similar increase after hormone treatment in the uptake of [^{32}P]phosphate by the liver did not accompany the enhanced incorporation of ^{32}P into RNA (Table 6). When rats were killed 10–16 hr.

Table 5. *Specific activities of rapidly labelled RNA in different cellular fractions and uptake of* [^{14}C]*orotic acid in livers of thyroidectomized rats at different times after the injection of tri-iodothyronine*

A 5·0 μc dose of [^{14}C]orotic acid was injected into each rat 10 min. before killing. The dose of tri-iodothyronine was 18 μg./100 g. body wt., each rat weighing 140 ± 7 g. No change in B.M.R. was detected until 25 hr. after hormone injection but there was a 16% increase at 42 hr. Each value is the average of two separate experiments with variations within ± 10%. Corrections were made for variations in body wt. and amount of isotope injected.

Time after tri-iodothyronine (hr.)	Uptake of [^{14}C]orotic acid (counts/min./g. of liver)	Specific activity (counts/min./mg. of RNA)			
		Nuclear RNA	Mitochondrial RNA	Microsomal RNA	Cell-sap RNA
0·0	88650	14400	175	308	462
3·3	84000	18300	206	401	425
6·4	86300	21000	160	480	575
11·0	95350	27600	231	415	661
16·1	138900	45250	205	516	823
21·0	159500	42000	322	500	776
42·0	141100	40780	365	559	695

Table 6. *Stimulation of incorporation* in vivo *of* [^{32}P]*phosphate into liver nuclear RNA, ribosomal RNA and s-RNA of thyroidectomized rats after the administration of tri-iodothyronine*

The 75 μc and 88 μc doses of [^{32}P]phosphate were injected into each rat 9·8 and 16·2 hr. respectively before killing. Other conditions were as in Table 5 and the text. Mitochondrial RNA values were unreliable because of large amounts of ^{32}P-labelled nucleotides and proteins recovered in these particles.

Time after [^{32}P]phosphate (hr.)	Time between tri-iodothyronine and [^{32}P]phosphate (hr.)	^{32}P content (μc/g. of liver)	Specific radioactivity (mμc of ^{32}P/mg.atom of RNA P)		
			Nuclear RNA	Ribosomal RNA	s-RNA
9·8	0·0	1·72	8·35	3·10	9·20
	6·5	1·60	13·91	3·05	11·00
	12·0	1·91	23·46	3·43	9·95
	25·2	2·06	26·60	4·95	13·18
16·2	0·0	1·28	6·68	5·15	6·74
	6·5	1·33	9·15	6·35	10·22
	10·0	1·19	12·50	7·60	10·34
	16·2	1·70	19·39	8·42	11·31
	21·0	1·66	17·83	12·11	12·91
	30·5	1·49	14·20	12·00	10·05

Table 7. *Base composition of nuclear RNA and ribosomal RNA labelled with* [^{32}P]*phosphate* in vivo *in normal, thyroidectomized and tri-iodothyronine-treated thyroidectomized rats*

Rats were killed 16·2 hr. after receiving 88 μc of [^{32}P]phosphate; 18 μg. of tri-iodothyronine was injected into one group of thyroidectomized rats 46 hr. before killing. RNA from thyroidectomized rats was extracted from the same preparations used in experiments shown in Table 6. Each value is the percentage of the total ^{32}P in the recovered four nucleotides (92–96% recovery from RNA hydrolysates).

Rats	RNA	Distribution of ^{32}P (%)				$\frac{(AMP+UMP)}{(GMP+CMP)}$ ratio
		AMP	GMP	CMP	UMP	
Normal	Nuclear	20·5	29·3	28·7	20·0	0·70
	Ribosomal	19·2	32·9	29·4	18·0	0·60
Thyroidectomized	Nuclear	21·2	30·0	27·8	20·2	0·71
	Ribosomal	19·8	32·2	30·1	17·9	0·61
Thyroidectomized + tri-iodothyronine	Nuclear	21·4	30·2	27·0	20·2	0·72
	Ribosomal	20·5	31·4	30·0	17·6	0·62

after the administration of [^{32}P]phosphate, ribosomal RNA accounted for the bulk of the radioactive RNA. In these long-term labelling experiments, administration of thyroid hormone resulted in a 2–8-fold higher specific activity of both the nuclear RNA and cytoplasmic RNA. However, the hormone did not alter the base composition of the newly synthesized RNA. In Table 7 is summarized the effect of tri-iodothyronine on the distribution of ^{32}P in the four nucleotides of nuclear and ribosomal RNA in two groups of animals shown in Table 6. The distribution of labelled AMP, UMP, GMP and CMP in ribosomal RNA from thyroidectomized rats agrees well with the base composition of ribosomal or bulk RNA in rat liver reported by other workers (Hiatt, 1962; Lipshitz-Wiesner & Chargaff, 1963; Lieberman & Kane, 1965). Much of the ^{32}P-labelled RNA from the nucleus was of the ribosomal type, with a high guanosine + cytidine content, although the (AMP + UMP)/(GMP + CMP) ratio was consistently 10–15% higher than in RNA extracted from cytoplasmic ribosomes. In two experiments in which animals were killed at 30 and 90 min. after giving [^{32}P]phosphate, this ratio was considerably higher and therefore more DNA-like. No conclusions could, however, be drawn in this case as to the effect of the hormone on account of the high degree of quantitative variability from one group of control animals to another.

The absence of any effect of thyroidectomy and tri-iodothyronine administration on the base composition of RNA synthesized *in vivo* was paralleled by experiments *in vitro* where the nearest-neighbour frequency of the RNA products of the two RNA-polymerase reactions was measured (Table 8). It has already been demonstrated that it is the Mg^{2+}-activated, and not the Mn^{2+}/ammonium sulphate-activated, RNA polymerase in liver nuclei that is initially stimulated by thyroid hormone administration (see Fig. 1). In the presence of ammonium sulphate the product is more DNA-like; the functional significance of the different RNA products of the polymerase is discussed by Widnell & Tata (1966*b*).

Table 9 depicts the net accumulation of ribosomal RNA as a function of time after hormone treatment. The values for total liver RNA and ribosomal RNA, as well as the RNA/protein ratio in microsomes (see Table 11), were decreased 4 weeks after thyroidectomy to 50–60% of those found in normal rats. These values were restored to nearly normal levels at about 45 hr. after a single injection of tri-iodothyronine, which reproduces previous observations under identical conditions (Tata *et al.* 1963; Roodyn *et al.* 1965). Net accumulation of newly formed RNA in cytoplasmic ribosomes was not clearly evident for about 24 hr. after the hormone although the synthesis of rapidly labelled nuclear RNA was accelerated within 6 hr. of hormonal

Table 8. *Nearest-neighbour base frequency of the product of the two RNA-polymerase reactions of liver nuclei isolated from normal, thyroidectomized and tri-iodothyronine-treated thyroidectomized rats*

Polymerase reaction	Radioactive nucleotide	Source of nuclei	Percentage of ^{32}P counts/min. recovered				Percentage recovery of ^{32}P counts/min. from column
			In UMP	In GMP	In CMP	In AMP	
Mg^{2+}-activated	[α-^{32}P]ATP	Normal rat	20	29	29	22	98
		Thyroidectomized rat	17	33	31	19	97
		Thyroidectomized rat, tri-iodothyronine-treated	18	33	30	19	102
	[α-^{32}P]GTP	Normal rat	19	34	30	17	97
		Thyroidectomized rat	19	35	30	16	100
		Thyroidectomized rat, tri-iodothyronine-treated	18	34	28	20	95
$Mn^{2+}/(NH_4)_2SO_4$-activated	[α-^{32}P]ATP	Normal rat	26	24	20	30	96
		Thyroidectomized rat	25	25	23	27	99
		Thyroidectomized rat, tri-iodothyronine-treated	27	25	20	28	98
	[α-^{32}P]GTP	Normal rat	34	27	10	29	101
		Thyroidectomized rat	37	24	9	30	94
		Thyroidectomized rat, tri-iodothyronine-treated	34	28	9	29	99

Table 9. *DNA, nuclear RNA, ribosomal RNA and s-RNA in livers of thyroidectomized rats at different times after a single injection of tri-iodothyronine*

Values were obtained from the liver preparations used for experiments in Tables 5 and 6.

Time after tri-iodothyronine (hr.)	DNA (mg./g. of liver)	Nuclear RNA (mg./mg. of DNA)	Ribosomal RNA (mg./mg. of DNA)	s-RNA (mg./mg. of DNA)
0·0	1·97	0·26	2·46	0·24
6·2	1·94	0·22	2·52	0·22
11·2	2·01	0·31	2·47	0·34
16	1·93	0·25	2·51	0·27
21	1·94	0·30	3·02	0·32
42·2	1·84	0·33	3·64	0·24
Normal, untreated	1·81	0·32	3·81	0·22

treatment. There was a small increase in the content of s-RNA but only a slight or no accumulation of RNA in the nucleus between 20 and 40hr. after tri-iodothyronine administration. No change was recorded during this period in the DNA/liver, and a slight fall in the DNA/g. of liver at 40hr., which is an indication of the hypertrophic action of the hormone. Insignificantly small amounts of [^{32}P]-phosphate and [^{14}C]orotic acid were incorporated into DNA in any of the experiments.

It may be concluded from the above studies on RNA synthesis *in vivo* that an early effect of thyroid hormone is to accelerate synthesis of RNA in the nucleus, consequently leading to the appearance of more newly formed RNA in cytoplasmic ribosomes or microsomes.

Effect of tri-iodothyronine in vivo *on hepatic polyribosomal sedimentation profiles.* Since polyribosomes are considered to be aggregates of ribosomes on m-RNA strands (Warner, Knopf & Rich, 1963; Noll, Staehelin & Wettstein, 1963; Munro, Jackson & Korner, 1964) analysis of polyribosomal profiles in sucrose-density-gradient centrifuging was used as an indication of the effect of the hormone on the concentration of m-RNA in the cytoplasm. Fig. 5 shows that the fraction of total liver ribosomal RNA recovered as polysomes was higher in thyroidectomized animals killed 42hr. after a single injection of tri-iodothyronine. The increases in hormone-treated animals over the controls, as judged from the extinction at 260mμ of fractions recovered in tubes 9–25, averaged 40–50%. At the same time, after hormone treatment, a higher amount of RNA sedimented through the 60% sucrose cushion as RNA associated with a lipid-rich fraction, presumably consisting of membranous structures. Sucrose-density-gradient centrifuging of ribosomes obtained by treating the mitochondria-free supernatant with different amounts of sodium deoxycholate showed that a higher detergent concentration was necessary to release the particles from the membranes after hormone administration.

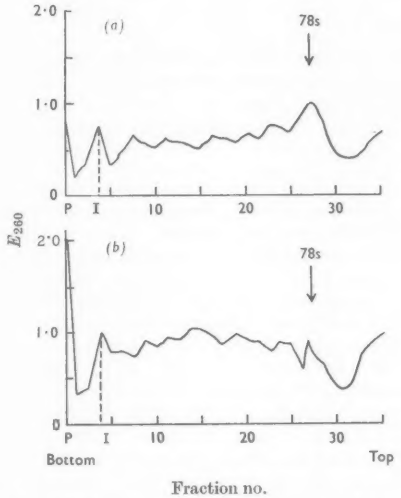

Fig. 5. Polyribosomal sedimentation profiles of 0·4% sodium deoxycholate-treated mitochondria-free supernatant from thyroidectomized rat livers. A 1·5ml. portion of the preparation was layered over discontinuous 15–30% sucrose density gradient with 3ml. of 60% sucrose at the bottom of the centrifuge tube. Centrifuging was for 1·8hr. at 22500 rev./min. in a Spinco SW25.1 rotor, and 1ml. fractions were removed and their extinctions measured as described in the text. (*a*) Untreated thyroidectomized rats; (*b*) thyroidectomized rats 45hr. after a single injection of 18μg. of tri-iodothyronine/100g. body wt. P indicates the pellet; I and the broken line indicate the interface between the 30% and 60% sucrose layers. The arrow indicates the position of 78s single ribosomes.

Briefly, the effect of thyroid hormone administration led to the reversion of polyribosomal sedimentation profiles and of the association of the particles to membranous structures to those found in normal animals.

Table 10. *Specific radioactivity of RNA in the 0·4%-sodium deoxycholate-resistant membranes, polyribosomes and monomeric ribosomes from livers of thyroidectomized rats with or without treatment with tri-iodothyronine*

All thyroidectomized rats were given 9·6μc of [^{14}C]orotic acid 2·2 or 15·2hr. before killing; 24μg. of tri-iodothyronine was injected 42·4hr. before death to thyroidectomized rats weighing 160$\pm$10g. Treatment of mitochondria-free supernatant preparation and sucrose-density-gradient centrifugation were as in Fig. 5. 'Membranes' correspond to the resuspended pellet in Fig. 5, interface to the zone included in fractions 1–4, polysomes to fractions 9–25, monomeric ribosomes to fractions 27–29 and the 'top' to fractions 31–35. RNA was estimated from E_{260} after correction for protein content. The ^{14}C determinations were carried out on individual fractions after the addition of 0·2mg. of carrier yeast RNA for precipitation.

Time after [^{14}C]orotic acid (hr.)	Time after tri-iodothyronine (hr.)	Specific activity of RNA (counts/min./E_{260})				
		'Membranes'*	Interface	Polysomes	Monomers	'Top'
2·2	0·0	1250	—	2100	750	<1000
	43·0	6700	—	3800	1200	<2000
15·5	0·0	5600	7000	11000	7800	<2000
	42·2	34500	14300	19800	17300	<4500

* The radioactivity recovered in the membranes from control animals was 5–8% of the total radioactivity put on the density gradient whereas it was 14–17% for the hormone-injected animals.

Radioactive-labelling experiments showed that the hormone-induced changes in polyribosome distribution were accompanied by the appearance of newly formed RNA in both the monomeric 78s ribosomes as well as their aggregates. The following facts emerge from Table 10: (*a*) The specific radioactivity of RNA in all regions of the sucrose-density-gradient profile was higher after hormone administration, in agreement with the ^{32}P-labelling experiments (see Table 7). (*b*) The 4–6-fold increase in specific activity of the RNA associated with lipid-rich constituents in hormone-treated animals, compared with a 2-fold stimulation in that of free polysomes and monomers, suggests that a larger proportion of newly formed m-RNA and RNP particles are firmly associated with microsomal membranes in preparations from hormone-treated animals than from the uninjected controls. There is also twice as much membrane-bound RNA in the livers of tri-iodothyronine-treated rats. (*c*) The specific radioactivity of RNA in polysomes 16hr. after administration of [^{14}C]orotic acid is only 10% higher than in the 78s monomers in both the treated and control animals. This suggests that an equilibrium has been achieved among the pre-existing and newly formed ribosomes and m-RNA, as suggested by Girard, Latham, Penman & Darnell (1965). There was also little variation (not exceeding $\pm$10%) in the specific radioactivity in polysomes of different sizes in both groups, which indicates an almost equal spacing of ribosomes along m-RNA strands of different length, in agreement with the conclusion of Marcus, Bretthauer, Halvorson & Bock (1965).

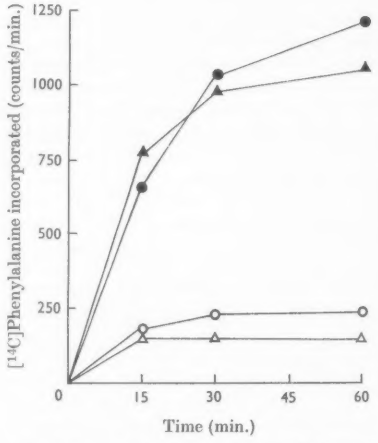

Fig. 6. Time-course of incorporation of [^{14}C]phenylalanine into trichloroacetic acid-soluble material by liver microsomes and RNP particles from thyroidectomized rats, in the presence and absence of polyU. ○ and ●, Microsomes; △ and ▲, RNP particles. ○ and △, PolyU absent; ● and ▲, incubation carried out in the presence of 100μg. of polyU/incubation tube.

Response of microsomes and ribosomes to synthetic m-RNA. The response of ribosomes to polyU and polyUG (mixed polymer of uridylic acid and guanylic acid) in the incorporation of phenylalanine and valine into protein has been used to evaluate

the effect of testosterone and growth hormone on the cytoplasmic m-RNA content of rat prostate (Liao & Williams-Ashman, 1962) and liver (Korner, 1964). We have applied the same principle to our studies on thyroid hormones. Since it has been reported that rat-liver microsomes and RNP particles may respond differently to polyU (Campbell *et al.* 1964; Kato, Loeb & Gelboin, 1965), both preparations were used under various conditions. In general, we were able to confirm the observations of Campbell *et al.* (1964) of the influence of polyU and Mg^{2+} concentrations on the incorporation of [^{14}C]phenylalanine into acid-insoluble material. However, the addition of polyU to both microsomes and RNP particles increased the duration of their ability to incorporate [^{14}C]phenylalanine (Fig. 6). The period of incubation used for comparing the stimulation by polyU of phenylalanine incorporation is therefore an important factor and the time-interval of 30min. was found to be a convenient one when dealing with both preparations.

Table 11 summarizes the results of an experiment in which treatment of thyroidectomized rats with tri-iodothyronine moderately decreased the ability of polyU to stimulate [^{14}C]phenylalanine incorporation by RNP particles but not by microsomes. The values of 7·72- and 5·61-fold polyU-directed stimulation of phenylalanine incorporation by RNP particles from untreated and treated rats respectively were reproducible and the difference was significant. In agreement with previous studies on the regulation of protein synthesis by thyroid hormones (Tata *et al.* 1963; Roodyn *et al.* 1965), thyroidectomy decreased the protein-synthesizing capacity of microsomes and ribosomes and tri-iodothyronine significantly increased it (by about 40%) about 40hr. after its administration. Again, no hormonal effect on cytoplasmic protein synthesis could be observed at 22hr. (It should be noted that the relative stimulation due to tri-iodothyronine was lower for the incorporation of [^{14}C]phenylalanine than that observed for [^{14}C]leucine, [^{14}C]-isoleucine and [^{14}C]valine.)

RNP particles, whether obtained with 0·4% or 1% deoxycholate, showed a greater response to polyU than did unfractionated microsomes, as was also observed by Campbell *et al.* (1964). A further addition of 6μmoles of magnesium chloride to the incubation medium markedly impaired in both preparations the extent of incorporation and decreased the polyU stimulation by 2–3-fold. The stimulation by polyU of RNP particles from thyroidectomized rats was decreased from 7·7-fold to 5·7-fold in particles from animals treated 42hr. earlier with tri-iodothyronine, the latter value being close to that found in normal animals. No such differences could be detected with microsomes, which underlines the difficulty in 'titrating' endogenous m-RNA concentration with polyU in the presence of membranous structure. Further, during the 42hr. after hormone administration, a marked increase had occurred in the cytoplasmic content of RNA, the microsomal RNA/protein ratio and firmer attachment of newly formed ribosomes to endoplasmic reticulum membranes (see Tables 9 and 10, Fig. 5 and the Discussion section).

Table 11. *Stimulation by polyU of the incorporation of* [^{14}C]*phenylalanine by liver microsomes and RNP particles from normal, thyroidectomized and tri-iodothyronine-treated thyroidectomized rats*

A 17μg. dose of tri-iodothyronine was injected/100g. body wt. 42hr. before killing. RNP particles were prepared by treating the mitochondria-free supernatant with 1% sodium deoxycholate. Each incubation vessel contained microsomes or RNP particles from 50mg. of liver and cell sap from 40mg. of liver; 125μg. of polyU was added wherever indicated. Other conditions are described in the text.

Rats	Preparation	RNA (mg./mg. of protein)	PolyU	Radioactivity in protein (counts/min./mg.)	Stimulation by polyU
Thyroidectomized	Microsomes	0·13	–	171	
			+	998	5·65
	RNP particles	0·26	–	447	
			+	3420	7·72
Thyroidectomized + tri-iodothyronine	Microsomes	0·19	–	235	
			+	1380	5·84
	RNP particles	0·36	–	599	
			+	3370	5·61
Normal, untreated	Microsomes	0·27	–	365	
			+	1908	5·51
	RNP particles	0·46	–	969	
			+	5232	5·43

Table 12. *Effect of some inhibitors of RNA and protein synthesis on the stimulation of B.M.R. and growth rate of normal and thyroidectomized rats*

Tri-iodothyronine and the inhibitors were injected twice at an interval of 48hr. The increase or decrease in the B.M.R. and body wt. 72hr. after the first injection were expressed as percentage changes from values recorded 1–2hr. before treatment was begun. Thyroidectomized rats weighed 150±10g. and normal rats of the same age were 235±15g. Each value is an average of two separate experiments with three rats/group. B.M.R. (ml. of O_2/g./hr.) was 1·46±0·10 and 1·05±0·06 for normal and thyroidectomized rats respectively; growth rate (increase in body wt./72hr.) was 17±3g. and 7±3g. for normal and thyroidectomized rats respectively. The dose of tri-iodothyronine was 24–27μg./100g. body wt. The doses of inhibitors/100g. body wt. were 8μg. of actinomycin D, 12mg. of puromycin, 5mg. of 5-fluorouracil and 10mg. of cycloheximide (7mg. for thyroidectomized rats).

	Normal rats			Thyroidectomized rats		
Treatment	ΔB.M.R.	ΔGrowth	Percentage inhibition*	ΔB.M.R.	ΔGrowth	Percentage inhibition*
None	± 2·7	+4·2	—	± 1·5	+ 5·4	—
Tri-iodothyronine	+16·5	+6·1	—	+35·3	+ 8·1	—
Actinomycin D	−12·6	−9·2	—	−14·0	−13·0	—
Tri-iodothyronine + actinomycin D	−10·0	−7·7	85	−13·8	−14·0	100·0
Puromycin	− 4·8	−6·3	—	+ 1·0	− 7·7	—
Tri-iodothyronine + puromycin	+ 4·0	−5·0	73	+ 9·8	− 6·0	69
5-Fluorouracil	−10·0	−8·8	—	−11·2	−13·2	—
Tri-iodothyronine + 5-fluorouracil	− 7·0	−8·4	79	− 9·0	−11·6	83
Cycloheximide	− 2·0	−1·5	—	0·0	− 5·0	—
Tri-iodothyronine + cycloheximide	0·0	−1·0	98	− 4·8	− 4·8	95

* Average of inhibition of increases in both B.M.R. and growth rate caused by thyroid hormone.

Effect of inhibitors of RNA and protein synthesis. In a previous report (Tata, 1963) it was shown that actinomycin D and puromycin were very effective in blocking the stimulatory effect of thyroid hormones on both the B.M.R. and growth rate. These studies have been extended to include two more inhibitors, 5-fluorouracil and cycloheximide, and the results are summarized in Table 12. All these inhibitors, which act at different steps of RNA and protein synthesis, were effective on normal as well as thyroidectomized rats treated with either thyroxine or tri-iodothyronine. The doses of inhibitors shown in Table 11 are those that gave the maximum inhibition of hormonal activity without producing a single fatality. Under these conditions, cycloheximide was the only substance that completely prevented the hormonal stimulation of B.M.R. and growth rate, presumably because it is a potent inhibitor of both protein and RNA synthesis (Wettstein, Noll & Penman, 1964; Fiala & Davis, 1965; Fukuhara, 1965). The relative timing of the administration of the inhibitor and the hormone was also an important factor in obtaining the antagonistic effects. For example, pretreatment of rats with the hormone 24hr. before the inhibitor considerably decreased the inhibition; pretreatment with actinomycin by more than 2 days before the hormone caused the animals to be refractory to the antibiotic in long-term studies. It is for this reason that the simultaneous administration of the antibiotic and the hormone in two doses spaced out by 48hr. was adopted and the same procedure followed for other inhibitors for the sake of standardization.

Table 13. *Effect of actinomycin D treatment* in vivo *on the stimulation of the* Mg^{2+}*-activated RNA-polymerase reaction of isolated liver nuclei by tri-iodothyronine*

Times of injection and doses of tri-iodothyronine and actinomycin D were as described in Table 12. The B.M.R. was determined 36hr. after the second injection of the two substances and nuclei were isolated for assay of RNA polymerase immediately afterwards.

Treatment of experimental animal			Specific activity of RNA polymerase ($\mu\mu$moles of [^{14}C]ATP incorporated into RNA/mg. of DNA)
Tri-iodothyronine	Actinomycin D	Relative B.M.R.	
−	−	100	621
−	+	87	596
+	−	147	1030
+	+	92	653

Actinomycin D is well known to be a potent inhibitor of DNA-dependent RNA polymerase in bacterial and mammalian systems (Goldberg & Rabinowitz, 1962; Hurwitz, Furth, Malamy &

Alexander, 1962). In view of the above results it was important to see what effect actinomycin D had on the hormonal stimulation of nuclear RNA synthesis, described above, under conditions in which the antibiotic abolished the physiological effects of the hormone. Table 13 shows that the same procedure of treatment with the antibiotic that blocked the growth-potentiation effect of two doses of tri-iodothyronine (Table 12) also inhibited the rise in hepatic RNA-polymerase activity that was observed after the same hormone treatment without having any effect on the activity of RNA polymerase when administered to control animals.

DISCUSSION

The relatively early effects of thyroid hormone on RNA synthesis in the nucleus could be detected well within the latent period preceding the hormonal stimulation of cytoplasmic protein synthesis (Tata *et al.* 1963; Roodyn *et al.* 1965). Although many immediate biochemical effects of thyroid hormones have been described (see Pitt-Rivers & Tata, 1959; Tata, 1964*a*), the accelerated synthesis of rapidly labelled nuclear RNA and the higher RNA-polymerase activity are the earliest effects *in vivo* under conditions in which both the B.M.R. and growth rate of most tissues would be stimulated.

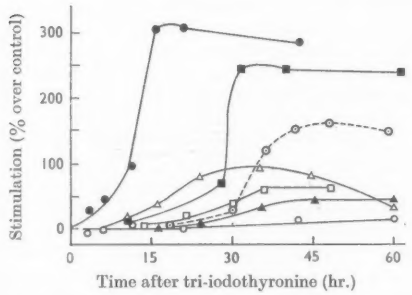

Fig. 7. Summary of data on the sequential stimulations of liver RNA synthesis, determined according to different parameters, in thyroidectomized rats after a single injection of 15–22 μg. of tri-iodothyronine/100g. body wt. In most cases, two or more parameters were determined in groups of six rats for each point on the curves. ●, Incorporation *in vivo* of [^{14}C]orotic acid into rapidly labelled nuclear RNA; △, Mg^{2+}-activated RNA-polymerase reaction in isolated nuclei; ▲, $Mn^{2+}/(NH_4)_2SO_4$-activated RNA-polymerase reaction in the same nuclei; □, mg. of ribosomal RNA/mg. of DNA; ■, incorporation of [^{32}P]phosphate into ribosomal RNA *in vivo* (9hr. after [^{32}P]phosphate administration); ○, mg. of nuclear RNA/mg. of DNA; ⊙--⊙, incorporation of ^{14}C-labelled L-valine, L-leucine or L-phenylalanine into protein by microsomes [this curve is based on combined data from Tata *et al.* (1963) and Roodyn *et al.* (1965)]. See the Discussion section for explanation.

In Fig. 7 we have summarized the sequential stimulations of RNA synthesis in the liver nucleus and cytoplasm as a result of a single injection of tri-iodothyronine into thyroidectomized rats. The first phenomenon observed was an increase in the specific activity of rapidly labelled nuclear RNA beginning at 3–4hr. after hormone administration, and reaching a peak value of 300% increase at about 16hr. Between these time-intervals a stimulation of Mg^{2+}-activated RNA-polymerase reaction was first recorded and this activity continued to rise until 42hr. after tri-iodothyronine was given. When, however, the polymerase was assayed in the presence of ammonium sulphate and Mn^{2+}, no hormonal effect was discernible for 24hr., after which only relatively small changes could be detected (45% as against 80% for the Mg^{2+}-activated system). A similar dissociation of the stimulations of RNA-polymerase activities in the presence and absence of ammonium sulphate has been observed, in our Laboratory and elsewhere, for growth hormone (Widnell & Tata, 1966*a*; Pegg & Korner, 1965) and oestrogen (Gorski, 1964; Hamilton, Widnell & Tata, 1965). A substantial rise in the amount of cytoplasmic ribosomal RNA and the microsomal RNA/protein ratio took place at about 24–30hr. after the hormone was given, thus elevating the hepatic ribosomal RNA content by 50–70%. (The concentration of hepatic ribosomal RNA in young thyroidectomized rats is 40–50% below that found in normal animals and consequently a single injection of thyroid hormone to non-thyroidectomized rats fails to produce the same dramatic increase in RNA content.) There was only a slight change in s-RNA concentration, and previous work (Tata *et al.* 1963) had shown that thyroid hormones under similar conditions did not affect the activation of amino acids or the capacity of cell sap to support protein synthesis by microsomes. No significant differences were recorded in the content of nuclear RNA. In view of the sustained increase in the synthesis of nuclear RNA (Tables 5 and 6) and the net accumulation of RNA in the ribosomal fraction (Tables 6 and 9), it may be concluded that thyroid hormone administration accelerated nuclear RNA synthesis and its transport into the cytoplasm. (The fraction of RNA synthesized in the nucleus that is transported into the cytoplasm under different conditions is not known.) The close association between the increase in cytoplasmic RNA and the higher amino acid-incorporating activity of cytoplasmic particles is interesting, although other factors besides RNA synthesis could also stimulate protein synthesis.

Sokoloff, Francis & Campbell (1964) have reported that thyroid hormones do not stimulate RNA

polymerase. This report was based on the observation that the addition of either nuclei or inhibitors of DNA-dependent RNA synthesis to a reconstituted rat-liver homogenate had no effect on the slight stimulation of amino acid incorporation that was induced by the addition of high concentrations of thyroxine *in vitro*. We have never observed any effect of the addition of thyroid hormone *in vitro* on the specific activity of RNA polymerase (see Table 4 and Widnell & Tata, 1963). In addition, the incorporation medium used by Sokoloff *et al.* (1964) contained neither CTP nor UTP, so that the incorporation of [^{14}C]AMP into RNA that they observed was more likely to represent terminal addition of ribonucleotides than actual RNA synthesis. Further, for the stimulation of amino acid incorporation into protein in a cell-free system by the addition of nuclei to have any significance, it would be necessary to demonstrate that RNA synthesized in the nucleus did stimulate amino acid incorporation by the cytoplasmic ribosomes present in the system, and no such evidence was presented by these authors.

Thyroidectomy or thyroid hormone administration did not alter the base composition of ^{32}P-labelled RNA *in vivo* or the nearest-neighbour frequencies in RNA formed by the Mg^{2+}-activated RNA-polymerase reaction (see Tables 7 and 8). In both cases the RNA synthesized was largely ribosomal in that it had a high guanosine + cytidine content, and a large part of rat-liver nuclear RNA is known to be ribosomal (Hiatt, 1962; Sporn & Dingman, 1963). Shorter ^{32}P-labelling periods than the ones reported above did give a more DNA-like RNA both in the nucleus and microsomes, as also noted by others (Hiatt, 1962; Munro *et al.* 1964; Trakatellis, Axelrod & Montjar, 1964), but the quantitative variations with short pulses of [^{32}P]-phosphate from one sample to another in the same group precluded any interpretation of hormonal effects.

A selective control of m-RNA synthesis by thyroid hormones could explain the specificity of their biological effects. Results of experiments on the response to polyU of the incorporation of [^{14}C]phenylalanine by microsomes and RNP particles (Table 11) and the polyribosome sedimentation profiles (Fig. 5 and Table 12) provide indirect but functional evidence for the subnormal cytoplasmic m-RNA content in the livers of thyroidectomized rats, which can be elevated by a single injection of tri-iodothyronine. However, the lowering of the response to polyU of RNP particles from thyroidectomized rats after tri-iodothyronine administration was not as marked as with ribosomes from the prostate stimulated with testosterone and from the liver with growth hormone (Liao & Williams-Ashman, 1962; Korner, 1964). A possible explanation for this difference may lie in the fact that the appearance of a large amount of new ribosomes, at the time after tri-iodothyronine administration at which polyU stimulation dropped from 7·7- to 5·5-fold, could lead to only slight increases in the overall m-RNA/ribosomes ratio in the cytoplasm.

The large increase in amino acid-incorporation activity per unit of microsomal RNA (Tata *et al.* 1963) suggests that the m-RNA or ribosomes or both synthesized after hormonal treatment is more active in protein synthesis than in the untreated animals. Since the sucrose-density-gradient analyses were limited to a few time-intervals after hormonal stimulation, it is not clear how the hormone would affect the stability and rates of entry and degradation of m-RNA and ribosomal RNA in the cytoplasm. Density-gradient analysis has also revealed a hormonal effect on the association between ribosomes or polysomes and microsomal membranes or some other lipid-rich component. The increase in RNA recovered as a pellet sedimenting through 60% sucrose in preparations from hormone-treated animals also meant that a higher detergent/RNA ratio was necessary to obtain the same degree of release of free particles. The specific-radioactivity distribution in the different gradient fractions suggests, further, a preferential attachment of newly synthesized RNA to the membrane-like fraction. A similar phenomenon has been observed in tadpole liver after thyroid hormone-induced metamorphosis (Tata, 1965). This disparity between 'old' and 'new' ribosomes does not suggest a constant exchange between free and bound ribosomes. With regard to the biological significance of the attachment of RNP particles to membranes of the endoplasmic reticulum, two recent studies have indicated that only those ribosomes attached to membranes represent the site of protein synthesis *in vivo* (Henshaw, Bojarski & Hiatt, 1963; Campbell *et al.* 1964).

That the four inhibitors of RNA and protein synthesis (actinomycin D, puromycin, 5-fluorouracil and cycloheximide) are powerful antagonists of thyroid hormone action in the intact animal enhances the biological implications of the present studies. Weiss & Sokoloff (1963) also reported the inhibition of the calorigenic action of thyroxine by puromycin but these workers interpreted this suppression as supporting evidence for a direct action of the hormone on the aminoacyl-s-RNA linkage to the polypeptide chain (Sokoloff, Kaufman, Campbell, Francis & Gelboin, 1963). Inhibition *in vivo* does not indicate the site of action of the hormone. Further, actinomycin D, which was initially used as an inhibitor of m-RNA synthesis *in vivo* (Reich & Goldberg, 1964), is an even more potent inhibitor of the synthesis of ribosomal RNA and conse-

quently of ribosomes (Perry, 1963; Franklin, 1963; Trakatellis *et al.* 1964). Similarly, puromycin and cycloheximide, which have largely been used for inhibiting cytoplasmic protein synthesis (see Darken, 1964), are also potent inhibitors of ribosomal RNA synthesis (Tamaoki & Mueller, 1963; Fiala & Davis, 1965; Fukuhara, 1965). Thus inhibitor studies in themselves only demonstrate that unimpaired RNA and protein synthesis is essential for the physiological activity of thyroid hormones.

The present results as a whole do not indicate the mechanism or site of action of thyroid hormones. However, stimulation of nuclear RNA synthesis is the earliest response to thyroid hormone under growth-promoting conditions and the sequential stimulation of cytoplasmic protein synthesis certainly indicates that control of RNA synthesis is one of the important initial steps in the cell leading to the diverse hormonal actions. Basically similar conclusions have been drawn for the developmental changes induced by thyroid hormones in amphibian metamorphosis (see Bennett & Frieden, 1962; Tata, 1965). A salient feature of these observations is that, although the synthesis of nuclear RNA is stimulated relatively early, the appearance of newly formed RNA in the cytoplasm is a slow process. The close association of increased protein-synthetic activity with the appearance of new ribosomes may perhaps reflect the transfer of 'precoded' ribosomes from the nucleus to the cytoplasm, as has been suggested for a bacterial system by Byrne, Levin, Bladen & Nirenberg (1964). Since the nucleolus appears to be the exclusive site of ribosomal RNA synthesis (see Prescott, 1964), it would be worth while to extend investigations on the possible regulation of nucleolar function by thyroid hormones. Whatever the site of action of thyroxine, the mechanisms of RNA transport and the specificity of ribosomal activity, our present results are compatible with the idea expressed elsewhere that thyroid hormones control not only growth and development but also B.M.R. and mitochondrial respiration via a selective control of protein synthesis (see Tata, 1964*a*; Tata *et al.* 1963; Lee & Lardy, 1965; Michels, Cason & Sokoloff, 1963; Roodyn *et al.* 1965; Gustafsson, Tata, Lindberg & Ernster, 1965).

Finally, almost all the effects of thyroid hormones on hepatic RNA metabolism described above have also been observed for a variety of growth and developmental hormones in their respective target tissues. Growth hormone, testosterone, oestrogen, adrenocorticotrophic hormone and ecdysone, soon after their administration, produce a 2–4-fold increase in the specific activity of rapidly labelled nuclear RNA (Talwar, Gupta & Gros, 1964; Korner, 1964; Wicks & Kenney, 1964; Bransome & Chargaff, 1964; Mueller, 1965; Gorski & Nelson, 1965; Sekeris, 1965) and a stimulation of RNA polymerase. Not surprisingly, actinomycin D, puromycin and cycloheximide are potent inhibitors of the hormonal action *in vivo* (Mueller, 1965; Hamilton, 1964; Gorski & Axman, 1964; Ferguson, 1963; Sekeris & Karlson, 1964). It was also found with testosterone acting on the seminal vesicles (Wicks & Kenney, 1964) and oestrogen on the uterus (Greenman & Kenney, 1964; Moore & Hamilton, 1964) that the hormonal stimulation of cytoplasmic protein synthesis accompanied the appearance of new ribosomes, although acceleration of nuclear RNA synthesis occurred much earlier. The simplest way to explain how the synthesis of different constituents is regulated by different hormones is to assume a specific de-repression of genes by each of these hormones. Nevertheless, the question arises whether the similarity of the biochemical phenomena produced by these hormones reflects some common site or route for the control of RNA synthesis in higher organisms. We have attempted to answer this question in the following paper (Widnell & Tata, 1966*a*).

We are grateful to Mr A. J. Bell and Mrs Helen Katz for technical assistance.

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Additive Effects of Thyroid Hormone, Growth Hormone and Testosterone on Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerase in Rat-Liver Nuclei

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(*Received* 26 *July* 1965)

1. The stimulations of DNA-dependent RNA polymerase in isolated rat-liver nuclei by thyroid hormone, human growth hormone and testosterone are compared. 2. Single or multiple administrations of growth-promoting doses of tri-iodo-L-thyronine, human growth hormone and testosterone stimulate the Mg^{2+}-activated RNA-polymerase reaction in nuclei from thyroidectomized, hypophysectomized and castrated rats respectively. The magnitude of stimulation was proportional to the degree of enhancement of liver growth by each hormone. After a single injection, the latent period preceding the stimulation was 1, 2 and 10 hr. for growth hormone, testosterone and tri-iodothyronine respectively. The time-course of stimulation of enzyme activity and the synthesis of rapidly labelled nuclear RNA *in vivo* were also different for each hormone. 3. Growth hormone administration failed to stimulate the Mn^{2+}/ammonium sulphate-activated RNA-polymerase reaction. Thyroid hormone and testosterone, however, stimulated it but the effect was less pronounced and occurred several hours later than that observed for the Mg^{2+}-activated RNA-polymerase reaction. 4. In combination experiments, hypophysectomized or the thyroidectomized rats were given growth hormone or tri-iodothyronine in a single or repeated doses at levels that produced the maximum stimulation of Mg^{2+}-activated RNA-polymerase activity. Taking into account the different latent period for each hormone, a single administration of the second hormone caused an additional stimulation of the enzyme activity. Similar additive effects were observed in thyroidectomized–castrated rats after treatment with tri-iodothyronine and testosterone. The magnitude of the additional stimulation caused by the administration of the second hormone was compatible with the capacity of that hormone to promote liver growth in rats deprived of it. 5. It is concluded that, although these hormones have some similar effects, the regulation of nuclear RNA synthesis may be mediated via different routes for each hormone.

In the preceding paper (Tata & Widnell, 1966) we demonstrated that the DNA-dependent RNA polymerase (EC 2.7.7.6) in liver nuclei (Weiss, 1960) was stimulated after the administration of thyroid hormones to thyroidectomized rats. The stimulatory effect was detected independently for both the Mg^{2+}- and Mn^{2+}/ammonium sulphate-activated RNA-polymerase reactions (Widnell & Tata, 1964*a*). The effects of thyroid hormones on RNA synthesis *in vivo* (Tata & Widnell, 1966) and the response of RNA polymerase were compatible both with the nuclear control of cytoplasmic protein synthesis and the regulation of protein synthesis by thyroid hormones (Tata *et al.* 1963; Roodyn, Freeman & Tata, 1965). In recent years, similar evidence has been obtained about the important role of nuclear RNA synthesis in the biological action of other mammalian growth and developmental hormones such as growth hormone, testosterone and oestrogen (Korner, 1962, 1964; Talwar, Gupta & Gros, 1964; Williams-Ashman, Liao, Hancock, Jurkowitz & Silverman, 1964; Kochakian, 1965; Mueller, 1965). The mechanisms by which hormones affect RNA metabolism are still unknown, but in view of the many similarities of phenomena observed, the question arises whether different hormones act through the same pathway or perhaps even on some common cellular sites. A primary requirement for answering this question would be to select a tissue that would depend on two or more hormones for its growth and development.

The marked dependence of the liver of young animals on both growth hormone and thyroid hormones for its growth and metabolic activity is

well known (see Simpson, Evans & Li, 1949; Pitt-Rivers & Tata, 1959; Tata, 1964). Although the major growth-promoting action of testosterone is manifested in the prostate and seminal vesicles, the growth of other tissues of the body, including the liver, is accelerated in young castrated animals after the administration of testosterone (see Kochakian, 1965; Frieden, 1964). The anabolic action of each hormone is not impaired when they are administered in combination, in that additive effects may be observed at the whole body and tissue levels. The administration of thyroid hormone and growth hormone to hypophysectomized–thyroidectomized rats has been shown to cause additive increases in body weight (Simpson, Asling & Evans, 1950), and additive effects have been observed in the salivary gland, a tissue that responds to both thyroid hormones and testosterone (Schafer & Muhler, 1960; Raynaud, 1964).

Recent evidence from several Laboratories suggests that the activity of DNA-dependent RNA polymerase in isolated nuclei reflects the growth rate of the tissue (Hancock, Zelis, Shaw & Williams-Ashman, 1962; Weill, Busch, Chambon & Mandel, 1963; Gorski, 1964; Tsukada & Lieberman, 1964; Tata & Widnell, 1966; Pegg & Korner, 1965). Therefore the aims of the present work were, first, to determine whether the administration of growth hormone and testosterone *in vivo* caused a stimulation of DNA-dependent RNA-polymerase activity in isolated liver nuclei. Secondly, we have investigated whether additive effects could be observed when thyroid hormone and either growth hormone or testosterone were administered in combination.

In addition, the DNA-dependent RNA-polymerase activity of isolated rat-liver nuclei can be resolved into two reactions (Widnell & Tata, 1964*a*). One reaction, the product of which was similar to ribosomal RNA, is actived by Mg^{2+}, whereas the second reaction, activated by Mn^{2+} in the presence of ammonium sulphate, yields a product that is more DNA-like (Widnell, 1965). These two reactions respond to thyroid hormone *in vivo* at different time-intervals after hormone administration (Tata & Widnell, 1966) and it was therefore decided to determine whether comparable differences might be observed for growth hormone and testosterone. A preliminary communication of these results has been presented (Widnell & Tata, 1964*b*).

EXPERIMENTAL

Animals. Male Sprague–Dawley rats were used in all the experiments. Thyroidectomy and castration were performed surgically when the animals weighed about 40g., and hypophysectomy at 100g., 3–4 weeks before the experiments. All hormones were injected subcutaneously.

Hormones. 3,3′,5-Tri-iodo-L-thyronine was obtained from Glaxo Laboratories, Greenford, Middlesex, and dissolved for injection as described by Tata & Widnell (1966). Human growth hormone (0·7i.u./mg.) was obtained from Dr D. R. Bangham of the Division of Biological Standards at this Institute, and was dissolved in 0·15M-NaCl immediately before injection. Testosterone propionate was obtained from Paines and Byrne Ltd., Greenford, Middlesex, as a

Table 1. *Effect of hypophysectomy and castration, and of the administration,* in vivo, *of growth hormone to hypophysectomized rats, and testosterone propionate to castrated rats, on the specific activity of RNA polymerase in isolated liver nuclei*

Experimental details are given in the text. The specific activity of the Mg^{2+}-activated RNA-polymerase reaction is expressed as $\mu\mu$moles of [^{14}C]ATP incorporated into RNA/15min./mg. of DNA, and that of the Mn^{2+}/$(NH_4)_2SO_4$-activated RNA-polymerase reaction as $\mu\mu$moles of [^{14}C]ATP incorporated into RNA/45min./mg. of DNA. Each value is the average of two or three separate determinations on nuclei extracted from livers pooled from three rats.

		Specific activity of RNA polymerase	
Experimental animal	Expt. no.	Mg^{2+}-activated	Mn^{2+}/$(NH_4)_2SO_4$-activated
(*a*) Normal, unoperated	1	886	2920
	2	942	3380
Hypophysectomized	1	464	1990
	2	476	2610
Hypophysectomized, treated with 200μg. of human growth hormone 3hr. before death	1	700	1980
	2	849	2620
(*b*) Normal, unoperated	1	1240	3490
	2	1160	2680
Castrated	1	865	2750
	2	715	1790
Castrated, treated with 200μg. of testosterone propionate 16hr. before death	1	1205	3310
	2	1010	2400

solution in ethyl oleate, and was diluted in arachis oil before use.

Experimental techniques. The isolation of rat-liver nuclei, the determination of the two RNA-polymerase reactions and NAD pyrophosphorylase, and the assay of DNA, RNA and protein, were as described by Tata & Widnell (1966).

RESULTS

Effects of individual hormones. It was shown in the preceding paper (Tata & Widnell, 1966) that both the Mg^{2+} and Mn^{2+}/ammonium sulphate-activated RNA-polymerase reactions of isolated rat-liver nuclei were lower than normal in thyroidectomized animals and were stimulated at about 12hr. and 30hr. respectively after a single injection of tri-iodothyronine. Preliminary experiments also showed that the specific activity of the two RNA-polymerase reactions was lower in either hypophysectomized or castrated rats than in the unoperated controls. The results presented in Table 1(*a*) show that the specific activity of the Mg^{2+}-activated, but not that of the Mn^{2+}/ammonium sulphate-activated, RNA-polymerase reaction was stimulated 3hr. after a single injection of growth hormone to hypophysectomized rats. Fig. 1(*a*) shows the time-course of the stimulation after administration of the hormones; a small but reproducible stimulation was observed after 1hr. and the maximum at 3hr. No stimulation of the Mn^{2+}/ammonium sulphate-activated reaction was observed at any time-interval tested, whereas the rapidly stimulated Mg^{2+}-activated reaction had returned to the control value during the 48hr. time-period investigated.

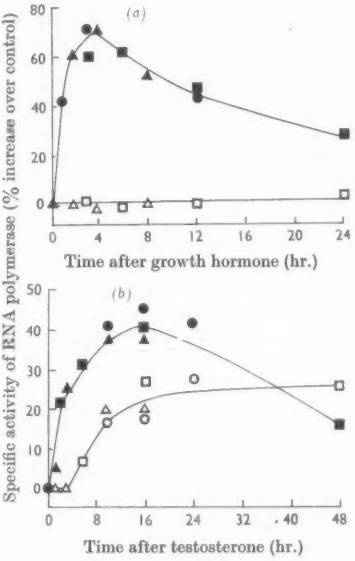

Fig. 1. Effect on the two RNA-polymerase reactions of isolated liver nuclei of a single injection of 200μg. of human growth hormone to hypophysectomized rats or 200μg. of testosterone propionate to castrated rats. The experimental details were as described in the text. ●, ▲ and ■, Mg^{2+}-activated RNA-polymerase reaction; ○, △ and □, Mn^{2+}/$(NH_4)_2SO_4$-activated RNA-polymerase reaction. Different symbols denote the values obtained from separate experiments. (*a*) Growth hormone administered to hypophysectomized rats; (*b*) testosterone propionate administered to castrated rats. Specific activities of the two RNA-polymerase activities were nearly the same as those in Tables 1, 2 and 5.

Table 1(*b*) shows that the specific activity of both RNA-polymerase reactions was stimulated 16hr. after a single injection of testosterone propionate to castrated rats. The time-course in Fig. 1(*b*) shows that, as with thyroid hormones (Tata & Widnell, 1966), the Mg^{2+}-activated reaction was stimulated before any change could be detected in the Mn^{2+}/ammonium sulphate-activated reaction. However, the latent period preceding the stimulation was shorter than that for thyroid hormones, in that the Mg^{2+}-activated reaction was stimulated within 2hr. and the Mn^{2+}/ammonium sulphate-activated reaction within 5hr. of hormone administration.

The stimulation of RNA polymerase as a function of the dose of growth hormone and testosterone administered to hypophysectomized and castrated rats respectively was comparable with the dose response for the effects on whole body growth (see Li, 1953; Kochakian, 1950). Fig. 2(*a*) shows that, after growth hormone administration, maximum stimulation of the Mg^{2+}-activated RNA-polymerase reaction was observed at a dose of 200μg. of growth hormone/100g. body wt.; no stimulation of the Mn^{2+}/ammonium sulphate-activated reaction was observed at any dose level. Fig. 2(*b*) shows the dose–response curves for the two RNA-polymerase reactions after testosterone propionate administration. For both the reactions the maximum stimulation was observed at a dose of 200μg. of testosterone propionate/100g. body wt.

As was also found for thyroid hormones (Tata & Widnell, 1966), the essential characteristics of both RNA-polymerase reactions, namely dependence on the presence of all four nucleoside triphosphates, DNA-dependence and the incubation time-course of the reaction, were unaffected by the endocrine status of the animals from which the liver nuclei were prepared. All three hormones were without effect on either RNA-polymerase reaction when added *in vitro* to nuclei obtained from hormone-deficient rats. No effects were observed on the specific activity of NAD pyrophosphorylase in the

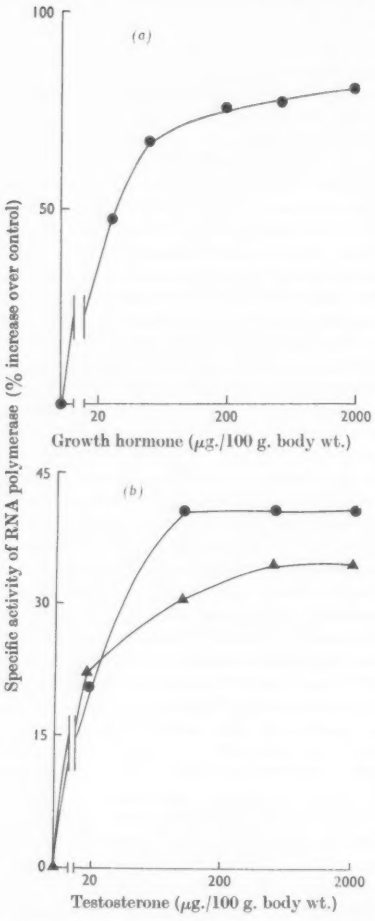

Fig. 2. Effect of the dose of human growth hormone administered to hypophysectomized rats, or testosterone propionate administered to castrated rats, on the two RNA-polymerase reactions of isolated liver nuclei. ●, Mg^{2+}-activated RNA-polymerase reaction; ▲, Mn^{2+}/$(NH_4)_2SO_4$-activated RNA-polymerase reaction. (*a*) Growth hormone administered to hypophysectomized rats 3 hr. before killing; (*b*) testosterone propionate administered to castrated rats 16 hr. before killing. Control values for untreated animals are shown in Tables 1, 2 and 5.

nuclear preparations from animals treated with the hormones *in vivo*, under conditions in which RNA polymerase was stimulated.

Additive effects of the hormones. In all the experiments described below on the effects of the hormones in combination, the dose of hormone administered, and the time-interval between the injection of the hormone and killing, were such that each hormone effected its maximum stimulation of RNA polymerase.

(*a*) Combination of thyroid hormone and growth hormone. The results of the first of the experiments involving a combination of hormones are shown in Table 2. The procedure consisted in giving daily injections for 8 days of either tri-iodothyronine or growth hormone to hypophysectomized rats. At the end of this period, these animals received a single injection of 25 μg. of tri-iodothyronine or 100 μg. of growth hormone 24 or 3 hr. respectively before being killed. These doses and time-intervals were selected on the basis of the effects observed for each hormone independently [see Tata & Widnell (1966) for thyroid hormone; Figs. 1(*a*) and 2(*a*) for growth hormone]. Both chronic and acute treatment with either hormone caused a stimulation of the Mg^{2+}-activated RNA-polymerase reaction, and when the rats receiving chronic treatment with one hormone were subjected to acute administration of the other an additional stimulation of the enzyme was observed. Further, the magnitude of the stimulation of RNA polymerase caused by acute treatment with one hormone was essentially the same whether or not the animals had received chronic treatment with the other.

Table 3 shows that the administration of single injections of either tri-iodothyronine or growth hormone to hypophysectomized rats caused a stimulation of the Mg^{2+}-activated RNA-polymerase reaction. When the hormones were administered together as single injections to the same animal, the effect was essentially additive, whether the animals were killed 24, 42 or 45 hr. after tri-iodothyronine, or 3 or 12 hr. after growth hormone. Stimulation of the Mn^{2+}/ammonium sulphate-activated reaction was only observed 42 or 45 hr. after tri-iodothyronine, and growth hormone was without effect on this reaction, in agreement with the findings reported in Table 1(*a*) and Fig. 1(*a*). Table 4 shows the results of a similar experiment in which thyroidectomized rats instead of hypophysectomized rats were used. Essentially the same results were obtained.

(*b*) Combination of thyroid hormone and testosterone. The results of two experiments in which thyroidectomized–castrated rats received chronic administration of tri-iodothyronine and testosterone propionate separately and in combination are shown in Table 5. The administration of either hormone caused a stimulation of both RNA-polymerase reactions, and when both hormones were administered the effect was essentially additive. As was observed with the combination of growth hormone and thyroid hormone (see Table 3), the

Table 2. *Effect of a single and of multiple doses of tri-iodothyronine and growth hormone on hypophysectomized rats as measured by the* Mg^{2+}*-activated RNA-polymerase reaction of isolated liver nuclei*

The specific activity of RNA polymerase was as expressed in Table 1. Three rats/group were used. Acute hormone treatment: a single injection of 25 μg. of tri-iodothyronine or 200 μg. of growth hormone was made at the time indicated before killing the animals. Chronic hormone treatment: 2 μg. of tri-iodothyronine or 25 μg. of growth hormone was injected daily for 8 days before killing the animals; the last dose of tri-iodothyronine was 24hr., and growth hormone 6hr., before killing. At the beginning of the experiment all animals weighed about 100g., and chronic treatment was for 8 days. Body growth and basal metabolic rate (B.M.R.) were expressed relative to the untreated control animals on the day of killing.

Tri-iodothyronine treatment	Growth hormone treatment	Body wt. (% increase)	B.M.R. (% increase)	Specific activity of RNA polymerase	ΔRNA polymerase: Due to tri-iodothyronine (acute)	ΔRNA polymerase: Due to growth hormone (acute)
—	—	—	—	404		
Acute (24hr.)	—	—	—	531	127	
—	Acute (3hr.)	—	—	656		252
Chronic	—	6	49	578		
Chronic	Acute (3hr.)	7	47	819		241
—	Chronic	13	11	658		
Acute (24hr.)	Chronic	11	9	789	131	

Table 3. *Effect of the administration of single injections of tri-iodothyronine and growth hormone to hypophysectomized rats, separately and in combination, on the activity of the two RNA-polymerase reactions of isolated liver nuclei*

In both experiments hypophysectomized animals were used. The dose of tri-iodothyronine was 25 μg. and of growth hormone 200 μg.; the rats weighed about 100g. The specific activity of RNA polymerase was expressed as in Table 1. Each value is the average of two determinations on nuclei from pooled livers (three rats/group).

	Time after single injection (hr.): Tri-iodothyronine	Time after single injection (hr.): Growth hormone	Specific activity of RNA polymerase: Mg^{2+}-activated	Specific activity of RNA polymerase: $Mn^{2+}/(NH_4)_2SO_4$-activated
Expt. 1	—	—	646	2040
	45	—	861	2710
	45	3	1245	2810
	—	3	955	2020
Expt. 2	—	—	556	2390
	24	—	732	2430
	24	12	1030	2410
	42	—	795	2920
	42	12	1090	3010
	—	12	876	2360

administration of a single injection of one hormone to rats maintained on multiple injections of the other caused a further stimulation of RNA polymerase (Table 6). Further, the magnitude of this additional stimulation of RNA polymerase by each hormone is compatible with their relative capacities to stimulate whole body growth.

Results obtained in this Laboratory have shown, both with thyroid hormone acting on rat liver [Tata & Widnell (1966) and Table 7] and oestrogen in the rat uterus (Hamilton, Widnell & Tata, 1965), that the stimulation of RNA synthesis *in vivo* preceded the stimulation of RNA polymerase in isolated nuclei. With growth hormone, however, there was an initial decrease in the specific activity of the rapidly labelled nuclear RNA followed by an increase at later time-intervals. The fall in specific activity of nuclear RNA preceded the stimulation of RNA polymerase, as also shown in Table 7. It is probable that there is not necessarily a correlation between RNA synthesis *in vivo* and the activity of RNA polymerase *in vitro*.

Table 4. *Effect of the administration of single injections of tri-iodothyronine and growth hormone to thyroidectomized rats, separately and in combination, on the activity of the two RNA-polymerase reactions of isolated liver nuclei*

The experimental details were as described in Table 3, except that thyroidectomized rats instead of hypophysectomized rats were used.

Time after single injection (hr.)		Specific activity of RNA polymerase	
Tri-iodothyronine	Growth hormone	Mg^{2+}-activated	$Mn^{2+}/(NH_4)_2SO_4$-activated
—	—	645	2090
24	—	987	2240
24	3	1385	2160
42	—	922	2895
42	3	1296	3010
—	3	959	2140

Table 5. *Effect of chronic administration of tri-iodothyronine and testosterone propionate on the activity of the two RNA-polymerase reactions of isolated liver nuclei*

The specific activity of RNA polymerase was expressed as in Table 1. At the beginning of the experiments all the operated animals weighed about 130g. Chronic treatment consisted of daily injections of the hormone for 12 days; the dose of tri-iodothyronine was 5μg./rat/day, and that of testosterone propionate 300μg. (Expt. 1) and 250μg. (Expt. 2)/rat/day. The last injections were given 24hr. before killing the animals. Nuclei from three pooled livers were used in each group.

		Chronic hormone treatment		Specific activity of RNA polymerase	
		Tri-iodothyronine	Testosterone propionate	Mg^{2+}-activated	$Mn^{2+}/(NH_4)_2SO_4$-activated
Expt. 1	Thyroidectomized rats	−	−	446	1650
		+	−	753	2420
		−	+	529	2130
		+	+	842	2850
	Unoperated control	−	−	871	2780
Expt. 2	Thyroidectomized castrated rats	−	−	412	1785
		+	−	761	2570
		−	+	493	2130
		+	+	819	2820
	Unoperated control	−	−	857	2750

Table 6. *Effect of chronic and acute administration of tri-iodothyronine and testosterone propionate to thyroidectomized–castrated rats on the activity of the two RNA-polymerase reactions of isolated liver nuclei*

The specific activity of RNA polymerase was expressed as in Table 1. At the beginning of the experiment all the animals weighed about 130g. Chronic treatment was the same as for Expt. 1 in Table 5. For acute treatment, the dose of tri-iodothyronine was 25μg. and that of testosterone propionate was 300μg.

Tri-iodothyronine treatment	Testosterone propionate treatment	Specific activity of RNA polymerase	
		Mg^{2+}-activated	$Mn^{2+}/(NH_4)_2SO_4$-activated
—	—	451	1825
Chronic	—	782	2810
—	Chronic	551	2350
Chronic	Chronic	858	3080
Chronic	Acute (10hr.)	829	3180
Acute (24hr.)	Chronic	878	2340

Table 7. *Increase in specific activity of rapidly labelled nuclear RNA* in vitro *and DNA-dependent RNA polymerase in isolated nuclei, as a function of time after a single injection of tri-iodothyronine or human growth hormone*

Tri-iodothyronine (18 μg./100g. body wt.) was administered to thyroidectomized rats and growth hormone (200 μg./100g.) to hypophysectomized rats. Six rats were used for each time-interval; of these, three were given 6 μc of [^{14}C]orotic acid/rat 10min. before killing and the other three were used for determining the specific activity of the Mg^{2+}-activated RNA-polymerase reaction. Values for hormone-treated animals are expressed relative to those found in the uninjected controls. Absolute values for uninjected (zero-time) controls were: rapidly labelled nuclear RNA: 29800 and 41500 counts/min./mg. of RNA for thyroidectomized and hypophysectomized rats respectively; Mg^{2+}-activated RNA polymerase: 540 and 485 $\mu\mu$c of [^{14}C]ATP incorporated into RNA/15min./mg. of DNA for the same two groups. Variations of values were within $\pm$10%.

Hormone	Time after injection (hr.)	Relative specific activity: Rapidly labelled nuclear RNA	Relative specific activity: RNA polymerase
Tri-iodothyronine	0	100	100
	4	115	99
	6	165	100
	10	195	105
	16	320	137
	24	305	162
Growth hormone	0	100	100
	1	70	136
	2	54	159
	3	96	178
	4	148	174
	6	138	179
	8	124	159

DISCUSSION

Hypophysectomy and thyroidectomy of young rats leads to a retardation or cessation of the growth of the liver and other tissues. The administration of growth hormone to hypophysectomized rats has been shown to stimulate liver growth (Simpson *et al.* 1949) and to increase the concentrations of liver RNA and protein (Geschwind, Li & Evans, 1950). Stimulation of amino acid incorporation into protein after growth hormone administration to rats has been demonstrated both *in vivo* (Korner, 1960) and in cell-free systems from the liver (Korner, 1959, 1961), and a stimulation of the incorporation of RNA precursors into RNA has been reported (Talwar, Panda, Sarin & Tolani, 1962; Iwamoto, Moriyama, Tetsuka & Miura, 1963; Talwar *et al.* 1964; Korner, 1964). The administration of small amounts of thyroid hormones (L-thyroxine and tri-iodo-L-thyronine) to thyroidectomized or hypophysectomized rats stimulates growth and increases the RNA and protein content of the liver (see Pitt-Rivers & Tata, 1959; Tata, 1964). Under conditions where whole body growth was stimulated, the administration of these hormones *in vivo* stimulated the incorporation of amino acids into protein both *in vivo* (Michels, Cason & Sokoloff, 1963) and in cell-free systems from the liver (Tata *et al.* 1963; Stein & Gross, 1962; Roodyn *et al.* 1965). We have also shown that these effects of the hormone on protein synthesis were preceded by a stimulation of nuclear RNA synthesis (Tata & Widnell, 1966). Castration of mice led to a decreased liver RNA/DNA ratio, which was restored to normal by testosterone administration (Kochakian & Harrison, 1962). It has also been found that administration of the hormone *in vivo* stimulated the incorporation of RNA precursors into RNA in the liver *in vivo* (Cantarow, Williams, Melnick & Patchkiss, 1958).

The present results, together with those of Tata & Widnell (1966), show that the Mg^{2+}-activated RNA-polymerase reaction of isolated rat-liver nuclei was stimulated by the administration *in vivo* of small doses of thyroid hormones, growth hormone and testosterone, and that the combination of thyroid hormone with either growth hormone or testosterone caused an additive stimulation. The Mn^{2+}/ammonium sulphate-activated RNA-polymerase reaction was stimulated by thyroid hormone and testosterone at time-intervals after hormone administration that were different from those observed for the Mg^{2+}-activated reaction [see Fig. 1(*b*); Tata & Widnell (1966)], and was not affected by growth hormone. The latent period for the response of either RNA-polymerase reaction

was different for growth hormone, testosterone (see Figs. 1*a* and 1*b*) and thyroid hormone (Tata & Widnell, 1966). The essential characteristics of each RNA-polymerase reaction, namely dependence on the presence of DNA and all four nucleoside triphosphates, and the quality of the RNA product, were unaltered by the endocrine status of the animals. The observation that no hormonal effect on NAD pyrophosphorylase could be detected suggests that the stimulation of RNA polymerase was not mediated by some general non-specific stimulation of nuclear activity.

Our results are generally in agreement with reports from other Laboratories on the effects of growth and developmental hormones on nuclear DNA-dependent RNA polymerase. Hancock *et al.* (1962) found that castration decreased, and testosterone administration *in vivo* stimulated, the DNA-dependent RNA-polymerase activity of an 'aggregate enzyme' preparation from prostatic nuclei; they also found that the effect of testosterone was less pronounced when the activity was measured in a medium of high ionic strength. Weill *et al.* (1963) have shown that oestradiol administration to chickens caused a stimulation of liver RNA polymerase, assayed in the presence of ammonium sulphate. Gorski (1964), however, was only able to detect stimulation of uterine RNA polymerase, at early time-intervals after the administration of oestrogen to ovariectomized rats, in the absence of ammonium sulphate. Pegg & Korner (1965) have also reported that they were able to detect a stimulation of enzyme activity in liver nuclei only in the absence of ammonium sulphate, after growth hormone administration *in vivo*. It has also been shown that the specific activity of the enzyme is greater in regenerating than in normal rat liver (Busch, Chambon, Mandel & Weill, 1962; Tsukada & Lieberman, 1964), and, together with hormonal effects, it can be said that the specific activity of RNA polymerase in various tissues provides an accurate reflection of the rate of tissue growth.

For several reasons, the effects of thyroid hormone, growth hormone and testosterone on RNA polymerase are compatible with the action of these hormones at the whole body level. First, the dose–response pattern of the stimulation of the Mg^{2+}-activated RNA-polymerase reaction is similar to that of the growth of the liver in the intact animal for each hormone. Secondly, the relative latent periods preceding the onset of the stimulation of the enzyme by the different hormones are consistent with those observed for whole body growth. Thirdly, the magnitude of the stimulation of RNA polymerase by the hormones administered, separately or in combination, is proportional to the relative capacity for promoting liver growth.

The mechanism of stimulation of RNA polymerase by hormones is not known but three possibilities could be invoked, separately or in combination, to account for the observations. The stimulation could be caused (*a*) by an allosteric interaction between the hormone and the enzyme, with the resulting conformational change that would increase enzyme activity (Monod, Changeux & Jacob, 1963), or (*b*) by some hormone-mediated effect on the DNA template, either increasing that fraction of the DNA active in RNA synthesis, or increasing the template efficiency of specific loci (Williams-Ashman *et al.* 1964), or (*c*) by the hormones inducing the synthesis of the RNA-polymerase protein, so that assay of the activity of the enzyme *in vitro* would reflect the quantity of the enzyme in the original tissue.

Reports from several Laboratories suggest that growth-promoting hormones, and even some hormones without any anabolic action, induce a general stimulation of RNA synthesis, particularly that of ribosomal RNA synthesis (Wool & Munro, 1963; Kenney & Kull, 1963; Talwar *et al.* 1964; Korner, 1964; Bransome & Chargaff, 1964; Moore & Hamilton, 1964; Wicks & Kenney, 1964; Tata & Widnell, 1966). In this context, the RNA-polymerase reaction in isolated rat-liver nuclei found in the present work to be stimulated by thyroid hormones, growth hormone and testosterone was the reaction that appeared to synthesize ribosomal RNA (Widnell, 1965). These observations focus attention on the possible importance of the synthesis of ribosomes in the mechanisms of action of growth-promoting hormones. At the same time, the additive effects suggest that, whatever the mechanisms by which thyroid hormone, growth hormone and testosterone stimulate RNA polymerase, the initial or fundamental site of action is probably different for each hormone.

We thank Dr M. P. Stack-Dunne for performing the hypophysectomies, and Mrs H. Katz and Mr A. J. Bell for skilled technical assistance.

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Methionine Synthesis by Extracts of *Salmonella typhimurium*

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(*Received* 12 *August* 1965)

1. Following the genetic studies by Smith (1961) and Smith & Childs (1963) with methionine auxotrophs of *Salmonella typhimurium*, methionine formation from homocysteine has been investigated with cell-free extracts of this organism. 2. As found with *Escherichia coli* (Woods, Foster & Guest, 1964), methyl groups are formed by an N^5N^{10}-methylenetetrahydrofolate reductase. They are then transferred to homocysteine by either a simple N^5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase or alternatively a cobalamin-dependent N^5-methyltetrahydrofolate–homocysteine methyltransferase. 3. *S. typhimurium* differs from *E. coli* in being able to synthesize significant amounts of cobalamin.

Escherichia coli has alternative enzymic mechanisms for the conversion of homocysteine into methionine (Woods, Foster & Guest, 1964). These mechanisms differ at a point subsequent to the creation of the methyl group as methyltetrahydrofolate (Guest, Foster & Woods, 1964*a*).

In one mechanism, methyl transfer to homocysteine from either N^5-methyl-H_4PtG‡ or N^5-methyl-H_4PtG_3 is catalysed by a cobamide-containing enzyme (Guest, Friedman, Foster, Tejerina & Woods, 1964*b*); essential cofactors for this reaction are SAM and $FADH_2$ (Rosenthal & Buchanan, 1963; Foster, Dilworth & Woods, 1964*a*). In the alternative reaction, specifically N^5-methyl-H_4PtG_3 is used as substrate by N^5-methyl-H_4PtG_3–homocysteine methyltransferase, an enzyme that apparently does not contain cobalamin (Foster, Tejerina, Guest & Woods, 1964*b*); this enzyme requires only Mg^{2+} ions and is not active with N^5-methyl-H_4PtG (Guest *et al.* 1964*b*). Both methyltetrahydrofolates are formed from the corresponding N^5N^{10}-methylene derivatives (in turn formed from serine via serine hydroxymethyltransferase) by the action of N^5N^{10}-methylenetetrahydrofolate reductase (Guest *et al.* 1964*a*). These relationships are summarized in Scheme 1. The cobalamin-dependent pathway, in contrast with the parallel cobalamin-independent pathway, can operate only when vitamin B_{12} is supplied exogenously to *E. coli*, since this organism does not synthesize significant amounts of the vitamin (Foster *et al.* 1964*b*; Woods *et al.* 1964). On the other hand, the apoenzyme of the cobamide enzyme can be activated by the addition of cobalamin to either the growth medium or ultrasonic extracts of the organism (Kisliuk & Woods, 1960; Guest & Woods, 1960; Takeyama, Hatch & Buchanan, 1961).

Mutation to loss of N^5-methyl-H_4PtG_3–homocysteine methyltransferase gives an organism requiring cobalamin or methionine for growth, whereas mutation to loss of the N^5N^{10}-methylenetetrahydrofolate reductase produces an organism responding to methionine alone (Woods *et al.* 1964).

Mutants of *Salmonella typhimurium LT*-2 with these phenotypes are also known and have been studied from a genetic standpoint by Smith (1961) and Smith & Childs (1963). These workers obtained evidence (from experiments using nutritional techniques, syntrophism, phage transduction and gene transfer mediated by colicine-determinants) that methionine formation from homocysteine in *S. typhimurium* is probably similar to that in *E. coli*. The object of the present work was to provide evidence at the enzymic level for the mechanism of homocysteine methylation in *S. typhimurium*.

MATERIALS AND METHODS

Organisms. E. coli strain 121/176 is an auxotroph derived from the prototrophic strain W (A.T.C.C. no. 9637) and requires either methionine or cobalamin for growth (Davis &

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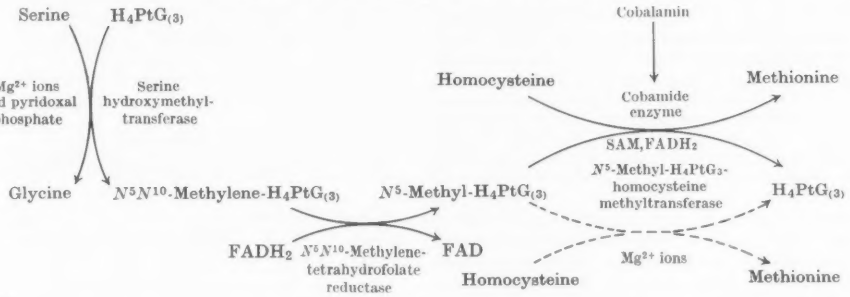
‡ Abbreviations: for members of the folic acid group, based on PtG for pteroylglutamate: H_4PtG, tetrahydropteroylmonoglutamate; H_4PtG_3, tetrahydropteroyltriglutamate; derivatives of these compounds are indicated as, e.g., N^5-methyl-H_4PtG (the term folate is used in this paper in reference to both simple and conjugated members of the folic acid family of vitamins); SAM, *S*-adenosylmethionine; EHC, extract of heated *E. coli*.



Scheme 1. Two pathways for the synthesis of methionine from homocysteine and serine in *E. coli*. $H_4PtG_{(3)}$ refers to either H_4PtG or H_4PtG_3. ⟶ indicates action with N^5-methyl-H_4PtG_3 only.

Mingioli, 1950). *E. coli* strain 3/62 requires methionine for growth and does not respond to either cobalamin or homocysteine; it was isolated from *E. coli* 518 (A.T.C.C. no. 9723) in this Laboratory by the technique of Adelberg & Meyers (1953). Strain 121/176 lacks N^5-methyl-H_4PtG_3–homocysteine methyltransferase, and strain 3/62 lacks N^5N^{10}-methylenetetrahydrofolate reductase (Foster *et al.* 1964*b*).

The auxotrophic strains of *S. typhimurium LT*-2 were obtained from Dr D. A. Smith. Strains *E*-197, *E*-205, *E*-47, *E*-230 and *E*-235 require either cobalamin or methionine for growth. Strain *F*-100 responds only to methionine (Smith & Childs, 1963). Strains *A*-15, *B*-23 and *C*-50 respond to either homocysteine or methionine, although not to cysteine. All organisms were maintained on slopes of nutrient agar (Oxo Ltd., London, E.C.4) subcultured monthly, incubated for 16 hr. at 37° and stored at 4°.

Organisms were grown on solid defined media in enamelled trays as described by Guest, Helleiner, Cross & Woods (1960). The basal medium was supplemented with DL-methionine (0·5 mM). Where indicated, cobalamin (15 mμmoles/500 ml. of medium) was used as a supplement alternative to methionine.

Ultrasonic extracts. They were prepared by the method of Guest *et al.* (1960) modified by the use of 20 mM-potassium phosphate buffer, pH 7·8, containing 2-mercaptoethanol (7 mM), for the preparation of suspensions of organisms before ultrasonic treatment. Extracts were stored at −20° and lost no activity over a period of 6 months.

The protein content of extracts was determined spectrophotometrically according to Layne (1957).

Extracts were treated so as to remove cofactors by gel filtration on Sephadex G-50 (Pharmacia Ltd., London, W.13) according to the method of Kisliuk (1960), and then by extraction with charcoal according to the method of Guest *et al.* (1964*b*).

EHC. An extract of heated *E. coli*, containing natural forms of folic acid, was prepared from acetone-dried organisms of strain PA 15 (grown in the absence of added cobalamin) as described by Guest *et al.* (1960). It is interchangeable with H_4PtG_3 as a cofactor in methionine synthesis by enzymic extracts of *E. coli*.

Tests for methionine synthesis. The conditions used for testing methionine synthesis were based on previous work with *E. coli* in this Laboratory. When serine was the ultimate source of C_1 units, the basal reaction mixture, M1, contained in a final volume of 1 or 2 ml.: potassium phosphate buffer (pH 7·8), 120 mM; DL-homocysteine, 6·7 mM; L-serine, 5 mM; ATP, 5 mM; glucose, 20 mM; $MgSO_4$, 5 mM; NAD, 0·5 mM; FMN, 0·25 mM; pyridoxal phosphate, 0·5 mM. Enzyme preparation was added equivalent to 1–10 mg. of protein/ml.; where the source of folic acid cofactor was EHC the equivalent of 14 mg. dry wt. of original organisms was used/ml. of reaction mixture.

The complete reaction mixtures were incubated at 37° for 1·5 hr. in an atmosphere of H_2 and the enzymic reaction was terminated by heating for 3 min. at 100°. The supernatant fluids, after centrifuging, were used for the assay of methionine by the microbiological method of Gibson & Woods (1960). DL-Methionine was used as standard; the organism responds only to the L-isomer. The value obtained (15–20 mμmoles) in each test for a control containing heated enzyme has been deducted from the experimental values recorded. No methionine degradation occurs under the incubation conditions since added L-methionine was completely recovered.

When [*Me*-^{14}C]methyltetrahydrofolate was the source of C_1 units, the incubation mixture, M2, contained in a final volume of 0·55 ml.: potassium phosphate buffer (pH 7·8), 55 μmoles; DL-homocysteine, 12·3 μmoles; 2-mercaptoethanol, 11·7 μmoles; $MgSO_4$, 2·5 μmoles. The additions made were: SAM, 1 mμmole; ATP, 200 mμmoles; FAD, 75 mμmoles; a $NADH_2$-generating system including NAD (250 mμmoles), ethanol (54 μmoles) and alcohol dehydrogenase (90 μg.); methylcobalamin, 5 mμmoles. Enzyme preparation equivalent to 2·5 mg. of protein was added. Substrates were either (±)-N^5[*Me*-^{14}C]-methyl-H_4PtG, 1·1 μmoles (0·37 μc), or (±)-N^5[*Me*-^{14}C]-methyl-H_4PtG_3, 1·6 μmoles (0·80 μc), per incubation volume.

The reaction mixtures were incubated at 37° in an atmosphere of H_2 for 30–45 min. The incubated reaction mixtures were passed through columns of Dowex 1 resin (X8; Cl^- form; 100–200 mesh) to remove unchanged folate as described briefly by Foster *et al.* (1964*a*). After washing the columns twice with 1 ml. of water, samples (0·2 ml.) of the combined effluent and washings were dissolved in 7 ml. of scintillation fluid containing 0·4% (w/v) of *p*-terphenyl

and 0·07% (w/v) of 2,2′-*p*-phenylenebis-(5-phenyloxazole) in toluene–ethanol–dioxan (12:5:3, by vol.). Radioactivity was estimated at 70% efficiency with an automatic tritium-scintillation counter (type 6020; Isotope Developments Ltd., Reading, Berks.). None of the materials present caused significant quenching of [*Me*-^{14}C]methionine. Methionine was the only radioactive product detected radioautographically after descending paper chromatography on Whatman no. 1 paper with as solvent butan-1-ol–acetic acid–water (4:1:5, by vol.; upper layer) or 2,4,6-collidine–2,5-lutidine–water (1:1:2, by vol.; upper layer). The amount of L-methionine formed was calculated from the initial specific activity of the methyltetrahydrofolate and agreed with values obtained by occasional random microbiological assays.

Chemicals. (±)-N^5[*Me*-^{14}C]-Methyltetrahydrofolates were prepared by reduction of (±)-N^5N^{10}-methylenetetrahydrofolates and purified by column chromatography as described by Guest *et al.* (1964*a*). Solutions in potassium phosphate buffer, pH 7·5 (50 mM), containing 2-mercaptoethanol (7 mM), were standardized spectrophotometrically (by using ϵ 28000 M^{-1} $cm.^{-1}$ at 290 mμ) and stored under H_2 at −20°. *S*-Adenosyl-L-methionine was isolated from yeast according to the method of Schlenk, Dainko & Stanford (1959). Methylcobalamin was a gift from Dr E. Lester Smith; crystallized cyanocobalamin was from British Drug Houses Ltd., Poole, Dorset. Crystallized yeast alcohol dehydrogenase was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. ATP, NAD, FMN and FAD were products of the Sigma Chemical Co., St Louis, Mo., U.S.A. Pyridoxal phosphate (Roche Products Ltd., Welwyn Garden City, Herts.) and DL-homocysteine (Mann Research Laboratories Inc., New York, N.Y., U.S.A.) were also commercial products.

Assay of cobalamin. The extracts were assayed for cobalamin with *Euglena gracilis* var. *bacillaris* (after release of the vitamin by heating and papain digestion) by the procedure of Foster *et al.* (1964*b*). Alternatively, a more rapid, though less sensitive, procedure of assay with *E. coli* 113/3 was used (Foster, Jones & Woods, 1961); in this case release of the vitamin was obtained by heating with HCl at 100° according to Ford, Holdsworth & Kon (1955).

RESULTS

Synthesis of methionine by extracts of methionine auxotrophs. Ultrasonic extracts of *S. typhimurium* *A*–15, *B*–23 and *C*–36 form methionine from homocysteine and serine when EHC is provided as the source of folic acid coenzyme (Table 1). This is to be expected since these strains are able to grow when supplied with homocysteine instead of methionine. As with *E. coli* (Szulmajster & Woods, 1960; Jones, Guest & Woods, 1961) synthesis is decreased if H_4PtG is added with EHC. However, in contrast with *E. coli*, H_4PtG does not give complete inhibition and is partially effective as a cofactor without added cobalamin (Table 1). The addition of cyanocobalamin to the extracts increases the synthesis with H_4PtG to that given by EHC.

Extracts of *S. typhimurium* *E*–47 (which requires either vitamin B_{12} or methionine) unexpectedly form methionine without the addition of cobalamin (Table 1), in contrast with results obtained by Guest *et al.* (1960) with an analogous cobalamin/methionine mutant of *E. coli* strain 121/176. Methionine formation with EHC as cofactor, or with H_4PtG, in the presence of cobalamin is, however, significantly less than that of the other strains under the same conditions. Strain *F*–100, the auxotroph with a growth response to methionine alone, formed only very small amounts of methionine with EHC as cofactor.

Table 1. *Synthesis of methionine by extracts of* S. typhimurium *auxotrophs*

Incubation was in mixture M1. Additions (per ml. final vol.) were: EHC, 0·1 ml.; H_4PtG, 1 μmole; cyanocobalamin, 0·74 mμmole.

	L-Methionine formed (mμmoles/mg. of protein/hr.) with			
Strain	EHC	EHC+ H_4PtG	H_4PtG	H_4PtG+ cyanocobalamin
A–15	66	49	48	66
B–23	53	37	30	50
C–36	38	27	22	36
E–47	16	20	19	19
F–100	5	—	—	—

Table 2. *Test for* N^5N^{10}*-methylenetetrahydrofolate reductase and* N^5*-methyl-*H_4*PtG–homocysteine methyltransferase in* S. typhimurium *auxotrophs*

Incubation was in mixture M1 with EHC as folic acid cofactor; each extract added was equivalent to 2·5 mg. of protein.

	L-Methionine formed (mμmoles/mg. of total protein)		
Strain	Alone	With *E. coli* 3/62	With *E. coli* 121/176
E. coli 3/62	8	—	200
E. coli 121/176	20	200	—
S. typhimurium *E*–197	30	130	24
S. typhimurium *E*–235	43	132	27
S. typhimurium *E*–47	100	215	58
S. typhimurium *E*–205	72	210	48
S. typhimurium *E*–230	75	210	46
S. typhimurium *F*–100	20	18	193

Synthesis of methionine by mixtures of cell-free extracts. *E. coli* strain 3/62, a methionine auxotroph, lacks N^5N^{10}-methylenetetrahydrofolate reductase; activation by test extracts of extracts of this strain to form methionine can be used to detect the reductase, e.g. in *E. coli* 121/176 (Guest *et al.* 1964*a*). Extracts of the cobalamin/methionine auxotrophs

of *S. typhimurium*, the *E* strains, increased methionine synthesis by extracts of *E. coli* 3/62 (Table 2), indicating that these strains contain the reductase. *S. typhimurium* strain *F*–100 does not do so, i.e. it lacks this enzyme.

Similarly, *E. coli* 121/176, a cobalamin/methionine auxotroph, lacks N^5-methyl-H_4PtG_3–homocysteine methyltransferase and, if vitamin B_{12} is absent from the test system, activation of extracts of this organism can be used as a test for this enzyme (Foster *et al.* 1964*b*). Extracts of the cobalamin/methionine auxotrophs of *S. typhimurium* have very little of this enzyme (Table 2), i.e. *S. typhimurium E* mutants are analogous in their metabolic lesion to *E. coli* 121/176. In strain *F*–100, on the other hand, the methyltransferase is present.

As would then be expected, mixtures of strains *E* and *F*, like mixtures of *E. coli* 3/62 and 121/176, formed methionine readily, e.g. extract of strain *F*–100 with *E*–235 made 160 mμmoles of L-methionine/mg. of protein/hr. and 266 mμmoles/mg. of protein/hr. with *E*–230.

The *S. typhimurium* cobalamin/methionine auxotroph can be divided into two genetic complementation groups (Smith & Childs, 1963). Of the strains studied, *E*–197 and *E*–235 are in one complementation group; *E*–47, *E*–205 and *E*–230 are in the other. Cell-free extracts might therefore exhibit complementation *in vitro* when incubated together. Significant increase of methionine synthesis, however, did not occur under these conditions (Table 3). On the other hand, different N^5N^{10}-methylenetetrahydrofolate-reductase activities in the two groups were observed (Table 2, penultimate column); this observation remains unexplained, since it is not clear how mutation to loss of the methyltransferase might affect the reductase activity, particularly when the two genes controlling the syntheses of these enzymes are not linked (Smith, 1961). If the mutation in the complementation group with low reductase activity were to produce a transmethylase so modified as to be enzymically inactive but still to bind strongly the small amounts of methyltetrahydrofolate formed at any one time from serine, then an inhibition of methionine formation would occur in the test for the reductase by admixture with *E. coli* 3/62. This hypothesis was tested by incubating extracts of mutants (*E*–225 and *E*–230) from each of the two genetic complementation groups in turn with mixed extracts of *E. coli* strains 121/176 and 3/62, i.e. providing both the reductase and methyltransferase from *E. coli*. However, the *S. typhimurium* extracts did not affect the methionine synthesis by the mixture of *E. coli* 3/62 and 121/176, indicating that the hypothesis is not correct.

Cobalamin and the methylation of homocysteine. (*a*) Organisms grown without added vitamin B_{12}. In *E. coli* methyl transfer from N^5-methyl-H_4PtG to homocysteine is catalysed by a cobamide-containing enzyme, which can be formed *in vitro* from methylcobalamin and an apoenzyme present in organisms grown without the vitamin. The action of this enzyme requires SAM as cofactor (Foster *et al.* 1964*a*).

Extracts of *S. typhimurium* also catalysed this methyl transfer (Table 4). However, in the absence of methylcobalamin, there was still considerable synthesis of methionine from N^5-methyl-H_4PtG, even with the cobalamin auxotrophs. This contrasted sharply with *E. coli*, where activity depends absolutely on the addition of a cobalamin. This synthesis, in the absence of methylcobalamin, was dependent on the addition of SAM and a reducing system. These requirements suggest strongly that the activity observed represented an endogenous

Table 3. *Complementation test* in vitro *with cobalamin auxotrophs of* S. typhimurium

Incubation was in mixture M1 with EHC as folic acid cofactor; each extract added was equivalent to 2·5 mg. of protein.

	L-Methionine formed (mμmoles/mg. of total protein) with strain				
Strain	*E*–197	*E*–235	*E*–47	*E*–205	*E*–230
E–197	30	36	62	44	58
E–235	36	43	66	60	60
E–47	62	66	100	86	80
E–205	44	60	86	72	74
E–230	58	60	80	74	75

Table 4. N^5-*Methyl-H_4PtG–homocysteine methyltransferase in extracts of* S. typhimurium

Extracts were from organisms grown with methionine as supplement and treated to remove cofactors. Incubation was for 45 min. in mixture M2 with N^5[*Me*-^{14}C]-methyl-H_4PtG. '$NADH_2$' refers to the $NADH_2$-generating system described in the Materials and Methods section.

	L-Methionine formed (mμmoles/mg. of protein/hr.) with extracts of strain			
Addition	*LT*–2	*E*–230	*E*–235	*F*–100
None	21	3	5	19
SAM	—	70	62	—
FAD, '$NADH_2$'	48	30	45	—
SAM, FAD, '$NADH_2$'	180	130	130	184
SAM, FAD, '$NADH_2$', methylcobalamin	220	220	190	220
FAD, '$NADH_2$', methylcobalamin	62	51	45	40

Table 5. *N^5-Methyl-H_4PtG_3–homocysteine methyltransferase in extracts of cobalamin/methionine auxotrophs of* S. typhimurium *grown with vitamin B_{12}*

Incubation was for 30 min. in mixture M2 with N^5-[*Me*-^{14}C]-methyl-H_4PtG_3. '$NADH_2$' refers to the $NADH_2$-generating system described in the Materials and Methods section.

Addition	L-Methionine formed (mμmoles/mg. of protein/hr.) with extract of strain	
	E–235	*E*–230
None	0	0
SAM	4	10
FAD, '$NADH_2$'	6	12
SAM, FAD, '$NADH_2$'	280	293
ATP, FAD, '$NADH_2$'	28	31
ATP, Mg^{2+}, FAD, '$NADH_2$'	280	285
SAM, FAD, '$NADH_2$', methylcobalamin	278	288

Table 6. *Methionine formation from N^5-methyl-H_4PtG_3*

Extracts were from organisms grown with methionine as supplement and treated to remove cofactors. Incubation was for 45 min. in mixture M2 with N^5[*Me*-^{14}C]-methyl-H_4PtG_3. '$NADH_2$' refers to the $NADH_2$-generating system described in the Materials and Methods section.

Addition	L-Methionine formed (mμmoles/mg. of protein/hr.) with extracts of strain	
	E–235	*F*–100
None	8	300
SAM	20	—
FAD, '$NADH_2$'	10	—
SAM, FAD, '$NADH_2$'	80	—
SAM, FAD, '$NADH_2$', methylcobalamin	250	380

cobamide holoenzyme (rather than a new enzyme similar to N^5-methyl-H_4PtG_3–homocysteine methyltransferase of *E. coli* but without the latter's folate specificity) since the cobamide enzyme of *E. coli*, when formed *in vivo*, requires $FADH_2$ as well as SAM for activity *in vitro*.

The results of Table 4 show that some cobamide apoenzyme is also present, since the further addition of methylcobalamin increases the methionine formed in the presence of SAM and the reducing system. Even in the presence of methylcobalamin, the omission of SAM still decreases methionine formation to the same extent.

(*b*) Organisms grown with added vitamin B_{12}. When *S. typhimurium E*–235 and *E*–230 were grown with vitamin B_{12} instead of methionine as supplement, there was no longer any effect of methylcobalamin *in vitro*, but there was now an absolute requirement for a reducing system (Table 5). Similar results were obtained with *E. coli*, where the cobamide enzyme, formed *in vivo*, requires after extraction reactivation by $FADH_2$ before participation in methyl transfer (Foster *et al.* 1964*a*). As with extracts from organisms grown without vitamin B_{12}, SAM was required and could be replaced by ATP and Mg^{2+}.

Occurrence of cobalamin in S. typhimurium. The results above with organisms grown without vitamin B_{12} suggested that, in contrast with *E. coli*, *S. typhimurium* has an endogenous vitamin B_{12}-dependent homocysteine methylation. This hypothesis was tested by measuring the vitamin B_{12} content of thick suspensions of *S. typhimurium* grown on unsupplemented medium (wild-type) or with methionine supplement (auxotrophs). The results (expressed as mμg. of cyanocobalamin/g. dry wt.) ranged from 300 to 1000 (measured with *Euglena*) or from 700 to 1100 (measured with *E. coli* 113/3). This is considerably more than that reported for *E. coli* (3 mμg./g. dry wt.) by Foster *et al.* (1964*b*). These authors calculated that the *E. coli* value corresponded to less than 0·5 mol. of vitamin B_{12}/cell. Correspondingly, *S. typhimurium* has then about 50–200 mol. of vitamin B_{12}/organism, i.e. contains a significant amount even when growing in a medium without cobalamin. It should be noted that the same quantity of cobalamin was found in the cobalamin auxotrophs as in the wild type.

Cobalamin-independent methylation of homocysteine. The studies with mixtures of cell-free extracts showed that the methionine auxotroph, strain *F*–100, contained N^5-methyl-H_4PtG_3–homocysteine methyltransferase. This was confirmed by direct assay with the defined substrate (Table 6). In contrast, extracts of the cobalamin auxotroph, *E*–235, did not catalyse methyl transfer from N^5-methyl-H_4PtG_3 to homocysteine unless supplemented with SAM, methylcobalamin and a reducing system, i.e. unless the cobalamin-dependent pathway was brought into operation. The addition of these cofactors to extract of strain *F*–100 was not necessary for methyl transfer from N^5-methyl-H_4PtG_3 compared with transfer from N^5-methyl-H_4PtG (see Table 4), but did enhance methionine formation to some extent.

DISCUSSION

Substantial methionine synthesis, comparable with that of *E. coli* (Foster *et al.* 1964*b*) and *Aerobacter aerogenes* (Morningstar & Kisliuk, 1965), was found to occur from homocysteine and serine with

ultrasonic extracts of *S. typhimurium*, except where these were derived from auxotrophs not responding to homocysteine for growth. The suggestion from the nutritional results (Smith, 1961) that enzymes similar to those in *E. coli* are present in *S. typhimurium* was borne out at the enzymic level. For example, H_4PtG is inhibitory to the action of EHC as folic cofactor but becomes, itself, a fully competent cofactor when vitamin B_{12} is added *in vitro* to vitamin-deficient extracts. Again, the formation of methyl groups appears to be via a methylene-tetrahydrofolate reductase as in *E. coli*, since an extract of *S. typhimurium* will activate an extract of *E. coli* that specifically lacks this enzyme; the reductase was absent from strain *F*–100, accounting for the methionine requirement of this strain.

The transfer of methyl groups to homocysteine occurs by two routes, as in *E. coli*. There is, for example in strain *F*–100, an enzyme that transfers methyl groups to homocysteine from N^5-methyl-H_4PtG_3 but not from N^5-methyl-H_4PtG. This did not require the addition of cofactors such as SAM and methylcobalamin, as did transfer from N^5-methyl-H_4PtG with the same strain. This apparently simple cobalamin-independent transmethylation was specifically lacking from the cobalamin/methionine auxotrophs. The division of these auxotrophs into two distinct complementation groups could not be interpreted as evidence for two separate enzymic steps in the methyl transfer reaction because no synergism was found between extracts from the two groups, although the production of fully competent extracts from the methionine auxotroph shows that any two such enzymes would be stable to extraction and assay. Further, no evidence was found for complementation *in vitro* arising from the recombination of enzyme sub-units.

In the cobalamin auxotrophs, there was a cobalamin-dependent methyl transfer, which was active with either N^5-methyl-H_4PtG or N^5-methyl-H_4PtG_3 and required SAM (and a source of reducing power if the cobalamin originated *in vitro*). These mutants were originally puzzling because their extracts were able to form methionine to the extent of 40% of that given by a competent strain with the source of natural folic acid as cofactor (Table 1), and yet these organisms showed no signs of 'leaky' growth in the absence of a methionine supplement (Smith, 1961). The results with the cofactor-dependent methyl transfer and the measurements of endogenous vitamin B_{12} indicate that *S. typhimurium*, and the cobalamin auxotroph in particular, differs from *E. coli* in being able to synthesize cobalamin to a limited, though significant, extent. Therefore this organism has a potential cobalamin-dependent methyl transfer that would operate even when the alternative vitamin-independent mechanism has been lost by mutation. Nevertheless, the vitamin synthesized is patently insufficient to satisfy the growth requirement. Calculations (Cauthen, 1965) have shown that growth of the cobalamin auxotrophs of *E. coli* (which does not synthesize vitamin B_{12}) ceases when the vitamin content of the cells falls to the levels found as endogenous material in *S. typhimurium*. The significance of the limited ability of *S. typhimurium* to synthesize cobalamin is not clear.

This research was aided by a grant to the Department from the Rockefeller Foundation and a grant towards expenses from the Medical Research Council.

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The Relationship Between Cellular Nucleic Acids in the Developing Rat Cerebral Cortex

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(*Received* 24 *August* 1965)

1. In the rat cerebral cortex net DNA synthesis ceases when the animal has reached about 25g. body weight (18 days of age). There is then little further change in the DNA content per cortex. 2. Nuclear and transfer RNA follow a similar pattern to DNA. 3. Microsomal and ribosomal RNA content increases up to 25g. body weight but then declines. The decrease in ribosomal and microsomal RNA content is associated with a change in RNA base composition. 4. Incorporation of [^{14}C]orotic acid into nuclear RNA proceeds at a similar rate in 4-day-old and adult animals. However, there is a lag period of about 60min. in the young animals during which incorporation into the ribosome fractions proceeds slowly. In the adult animals the lag period is not seen.

There have been many observations supporting the hypothesis that ribosomal RNA is first formed in the nucleus. According to McCarthy, Britten & Roberts (1962) in bacterial systems the newly formed RNA enters the cytoplasm in the form of 8–20s sub-units (eosomes), complete ribosomes subsequently being formed by aggregation. In a review of the literature Prescott (1964) concluded that ribosomal RNA arises solely from nuclear precursors. The principal opposition to this view has come from H. Harris and his group (Harris, 1959; Watts & Harris, 1959; Harris & Watts, 1962; Harris, Fisher, Rodgers, Spencer & Watts, 1963; Watts, 1964), who, working mainly with HeLa cells, have concluded that the rapidly synthesized nuclear RNA is rapidly broken down *in vitro* and find little or no evidence that it is transferred directly into the cytoplasm. Singh & Koppelman (1963) claimed that the results of Harris and co-workers were in fact compatible with a precursor relationship between nuclear and cytoplasmic RNA, but this was disputed by Harris (1963).

Much of the evidence for a nuclear RNA–cytoplasmic RNA precursor relationship is based on observations that labelled precursors enter nuclear RNA very rapidly whereas there is a lag period before the label appears in ribosomal RNA. This lag period appears to be extremely variable and may be only a matter of a few minutes in *Neurospora* (Zalokar, 1960) to several hours in cultured *Triturus* cells (Prescott, 1964). Prescott (1964) has suggested that the length of the lag period increases from cell to cell roughly in proportion to the length of the cell life cycle. Watts (1964) has also shown that the lag period can be altered by external factors. It could virtually be abolished in HeLa cells by allowing them to grow in a purine-free medium for 24hr. before precursor administration.

So far, however, very little work appears to have been done on the relationship between cellular nucleic acids in adult mammalian tissue. Adams (1965) in a study of [^{14}C]orotic acid incorporation into weanling and young-adult rat-brain RNA found that, when injected intracisternally, this compound became rapidly incorporated into all RNA fractions. The data suggested that there was little if any lag period between nuclear and ribosomal labelling.

The present paper describes further experiments related to this observation.

MATERIALS AND METHODS

Animals. Wistar albino rats bred in this Institute were used when 3–4 days of age (weight about 7g.) or as adults weighing 200–250g. In one experiment rats of intermediate sizes were also used.

Radioactive isotope. [^{14}C]Orotic acid was obtained from The Radiochemical Centre, Amersham, Bucks. It was diluted before use with unlabelled material to a specific activity 10μc/μmole, dissolved in 0·9% NaCl and injected into the subarachnoidal space (Lindberg & Ernster, 1949). Normally 4-day-old rats received 2μc (0·02ml.) and adults 4μc (0·04ml.).

Preparation of homogenates and subcellular fractions. Rats were killed by decapitation, the brains rapidly removed and the cerebral cortices dissected out. Material from three to five rats was pooled for each determination. Homogenization was effected by placing 1 part of the brain

together with 10–20 parts of 0·32M-sucrose containing 0·5mM-$MgCl_2$ (pH 7·2) in a glass homogenizer with a Teflon pestle, and rapidly moving the pestle up and down nine times. The fractions were collected by centrifuging as follows:

(1) 1000*g* for 5 min. in a Mistral 6L refrigerated centrifuge (nuclear fraction). The pellet was gently resuspended in 10ml. of fresh sucrose and recentrifuged, the supernatant being discarded.

(2) The original supernatant from (1) was centrifuged at 10000*g* for 10 min. in the no. 30 head of a Spinco model L ultracentrifuge, and the pellet discarded.

(3) The supernatant from (2) was centrifuged in the Spinco ultracentrifuge at 20000*g* for 1 hr. The pellet (microsome fraction) was resuspended in fresh sucrose and recentrifuged.

(4) The supernatant from (3) was again centrifuged in the Spinco ultracentrifuge (40 head) at 105000*g* for 2 hr. (ribosome fraction), the soluble fraction remaining in the supernatant.

The pellets were resuspended in 0·9% NaCl and stored at −20° until they were processed.

Extraction of DNA and RNA. This was done by the hot 10% NaCl method of Tyner, Heidelberger & LePage (1953), the lipid-extraction step being modified according to Adams (1965). The extracted nucleic acids were washed three times at −5° with ethanol (90%, v/v). All fractions were then submitted to mild alkaline hydrolysis in 0·1N-NaOH at 37° for 24 hr. and then made 0·1N with respect to HCl. After standing at room temp. for 1 hr. the preparations were centrifuged at 3000*g* for 10 min. and any precipitate was dissolved in 0·1N-NaOH and estimated for DNA. Normally DNA was found only in the nuclear fraction.

Estimation of nucleic acids. RNA was estimated by the orcinol method (Hurlbert, Schmidt, Brumm & Potter, 1954) and DNA by the diphenylamine reaction (Dische, 1955).

RNA base composition. This was determined by heating the alkaline hydrolysates of RNA in N-HCl for 1 hr. at 100°. Samples were run on paper (Whatman no. 1) for 48 hr. with 70% (v/v) 2-methylpropan-2-ol made 0·8N with respect to HCl as solvent. The adenine, guanine, uridylic acid and cytidylic acid spots were detected under ultraviolet light, cut from the paper and eluted with N-HCl at 38°.

The resulting solutions were read at their respective absorption maxima in a Unicam SP.500 spectrophotometer, and are uncorrected.

RESULTS

Age variation of the nucleic acid content of the cerebral cortex. The DNA content, and the RNA content of the various cell fractions, was estimated at intervals from 3 to 4 days of age (7g. body wt.). The average content per cortex of the various nucleic acids and the cortex weight, plotted against total body weight, are given in Fig. 1. This shows that the total DNA and nuclear and transfer RNA present per cortex increased up to about 25g. body wt. and then remained relatively constant. The ribosomal and microsomal RNA content also increased up to 25g. but then declined.

Orotic acid-incorporation studies. Fig. 2(*a*) shows

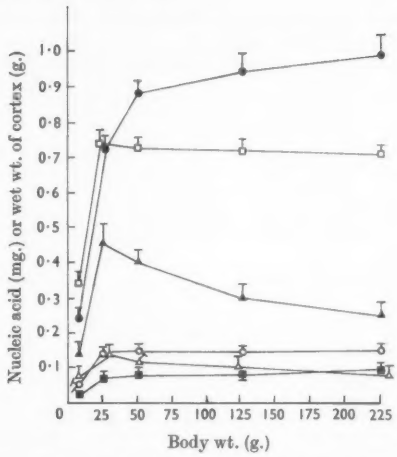

Fig. 1. Wet weight, DNA content and RNA content of subcellular fractions of the rat cerebral cortex during development. The nucleic acid values are the average amounts present in one whole cortex taken from rats of the stated body weights, as arithmetic means + S.E.M. (bars). Each point corresponds to six to eight estimations. ●, Wet wt. (g.); □, DNA content (mg.); ▲, ribosomal RNA content (mg.); △, microsomal RNA content (mg.); ○, nuclear RNA content (mg.); ■, transfer RNA content (mg.).

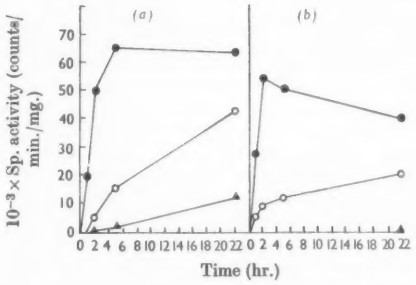

Fig. 2. Incorporation of [^{14}C]orotic acid label injected subarachnoidally into nucleic acids of the rat cerebral cortex. (*a*) 4-day-old rats (given 2μc); (*b*) adult rats (given 4μc). ●, Nuclear RNA; ○, ribosomal RNA; ▲, DNA.

the specific activity of RNA and DNA isolated from 4-day-old rat cerebral cortex after the injection of 2μc of [^{14}C]orotic acid, and Fig. 2(*b*) shows the results of a similar experiment in which adult rats (wt. 200–250g.) were injected with 4μc of [^{14}C]orotic acid. The labelling of the ribosomal

RNA in both groups over the first 2hr. is shown on an expanded scale in Fig. 3.

In the adult animals a very slow incorporation of label into DNA was observed, but this reached only 200 counts/min./mg. after 24hr. compared with 12000 counts/min./mg. in the young.

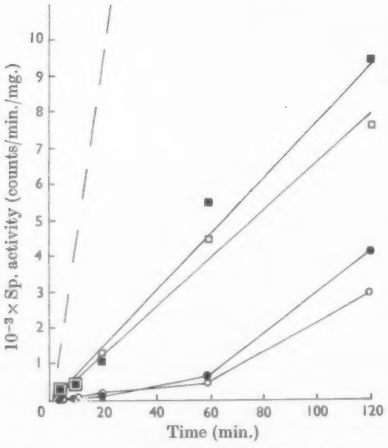

Fig. 3. Incorporation of [^{14}C]orotic acid into the ribosomal and microsomal RNA of rat cerebral cortex over the 5–120min. period. Details are as given in Fig. 2. Incorporation into nuclear RNA is represented by the broken line. Adult rats: ■, ribosomal RNA; □, microsomal RNA. 4-day-old rats: ●, ribosomal RNA; ○, microsomal RNA.

The ratios of incorporation into nuclear RNA/incorporation into ribosomal RNA for the two groups over the whole period are given in Table 1, and show that over the first 60min. this ratio was of the order of 40 for the young animals and 6 for adults. It seemed possible that this difference might be due to differences in rates of diffusion of label into the brain mass. To check this possibility adult rats were injected intracisternally with 4μc of [^{14}C]orotic acid, and the nuclear RNA/ribosomal RNA incorporation ratio was determined in cerebral cortex, cerebellum and white matter. Adams (1965) has shown that, when this route of injection was used, much more of the injected material becomes available to the cerebellum and white matter, than to the cerebral cortex, and the rates of diffusion into the three types of tissue should therefore be very different.

The incorporation into nuclear RNA and ribosomal RNA and the nuclear/ribosomal ratio over the 5–20min. period are shown in Table 2. Although there was a considerable difference in the specific activities of the three RNA samples the incorporation ratio was reasonably constant and similar to the adult figures in Table 1.

RNA base composition. In view of the observation (Fig. 1) that the ribosomal and microsomal RNA content of the cerebral cortex decreased by about

Table 1. *Ratios of incorporation of* ^{14}C *into rat-brain nuclear-fraction RNA to incorporation into microsoma-fraction RNA (N/M) or ribosomal-fraction RNA (N/R) after subarachnoid injection of* [^{14}C]*orotic acid*

	Time after injection						
	5 min.	10 min.	20 min.	60 min.	2 hr.	5 hr.	24 hr.
N/M							
Adult	5·6	6·4	8·0	7·5	7·3	4·0	1·7
4-day-old	18	34	41	36	12	4·6	1·8
N/R							
Adult	5·4	5·8	10·0	5·8	5·8	3·3	1·6
4-day-old	40	47	53	32	16	5·2	2·1

Table 2. *Incorporation of intracisternally injected* [^{14}C]*orotic acid into nuclear (N) and ribosomal (R) RNA of adult rat cerebral cortex, cerebellum and white matter*

Results are given in counts/min./mg. of RNA. 4μc (10μc/μmole) of orotic acid was injected into each rat.

	Time after injection								
	5 min.			10 min.			20 min.		
	N	R	N/R	N	R	N/R	N	R	N/R
Cerebral cortex	140	25	5·6	260	50	5·2	620	85	7·3
Cerebellum	350	80	4·4	1100	150	7·3	2700	325	8·3
White matter	450	70	6·4	780	100	7·8	1950	250	9·8

Table 3. *Base composition of RNA samples isolated from rat cerebral cortex*

Results are given as moles/100 moles ± S.E.M. and are uncorrected for guanine hydrolysis. The numbers of observations per figure are given in parentheses. The differences between the guanine and cytosine contents of the ribosomes from adult and 4-day-old animals are highly significant ($t > 4$ for 21 degrees of freedom).

	Guanine	Adenine	Cytosine	Uracil
Nuclear				
Adult (8)	30·5 ± 0·67	24·1 ± 0·42	26·4 ± 0·59	19·0 ± 0·48
4-day-old (10)	30·7 ± 0·42	24·2 ± 0·59	25·2 ± 0·77	19·9 ± 0·50
Microsomal				
Adult (10)	31·7 ± 0·49	19·5 ± 0·35	29·6 ± 0·47	19·2 ± 0·67
4-day-old (10)	33·6 ± 0·47	20·5 ± 0·18	27·9 ± 0·50	18·0 ± 0·31
Ribosomal				
Adult (13)	31·2 ± 0·35	19·7 ± 0·34	30·0 ± 0·37	19·1 ± 0·68
4-day-old (10)	33·7 ± 0·59	21·0 ± 0·38	27·7 ± 0·54	17·6 ± 0·62

40% as the animals increased in body weight from 25 to 225 g. estimations of base compositions were made in 4-day-old and adult animals. The base composition of nuclear RNA was also determined in the two groups, and the results are given in Table 3.

DISCUSSION

The results of Fig. 1 have shown that in the rat cerebral cortex net DNA synthesis ceased comparatively early (about 18 days of age, 25 g. body wt.). The net synthesis of all cellular RNA ceased at about the same point, and this was followed in the microsomal and ribosomal RNA by a decrease of nearly 40% by the time the animals reached 200–250 g. body wt. It seems reasonable to suppose that this RNA decrease represents a decline in the microsome–ribosome population.

The subsequent studies were therefore made on the cerebral cortex of 3–4-day-old rats in which net synthesis of all nucleic acids was proceeding, and of adult (200–250 g.) rats in which there was no net synthesis and a declining microsome–ribosome population. Preliminary experiments with [^{14}C]-orotic acid showed that if the adult animals were injected with 4 μc and the 4-day-old animals with 2 μc the incorporation of label into the nuclear RNA of both groups was approximately linear and at the same rate for 2 hr. Since the cortex of the adult was three to four times as large as that of the 4-day-old, this suggested that there was no great age difference in ability to incorporate label into nuclear RNA.

The rapidity and similar rate of the nuclear incorporation in both groups suggests that most of the nuclear RNA is undergoing a rapid intracellular turnover, particularly in the adult animals in which there was no net synthesis of RNA. This is in substantial agreement with the work of Watts (1964).

In each group the pattern of incorporation of label into microsomal and ribosomal RNA was very similar, but very different in the two groups. In the 4-day-old animals there was very little ribosomal labelling in the first 60 min. and then a rapid increase, whereas the ribosomal labelling in the adults was essentially linear from 5 to 120 min. and when extrapolated cut the time axis at +3 min., which is closely similar to the intersection of the plot of the nuclear incorporation. These results suggest that the lag period between nuclear and ribosomal incorporation is abolished in the adult cells. The interpretation of the results is complicated by lack of knowledge of the nature of the labelled RNA associated with the ribosomes, in particular whether it is all of a 'messenger' type in the two groups. However, the results obtained with young brain seem consistent with the usual view of a precursor relationship between nuclear and cytoplasmic RNA. A similar conclusion has recently been reached by Singh (1965) through a kinetic analysis of precursor incorporation into young brain. The situation in the adults certainly seems dissimilar, and the results suggest two possibilities. Either in the adults the ribosomal RNA has become self-replicating, or precursors are entering the cytoplasm from the nucleus at a greatly increased rate. However, in view of the declining ribosome population in the adult this second alternative seems less likely.

The results of Watts (1964) showing that the lag period between nuclear and cytoplasmic incorporation in HeLa cells could be abolished by preincubation of the cells in a purine-free medium were interpreted by this author in terms of a diminished intracellular purine pool size permitting more rapid precursor incorporation into cytoplasmic RNA. This possible reason for change in lag period seems unlikely to bear on the present results since the total uridine nucleotide pool is only about 20% lower in the adult than in the newborn rat cerebral cortex (Adams, 1965).

The base composition of nuclear RNA from both young and adult cortex was similar and the RNA

contained 56–57% of guanine–cytosine. Microsomal and ribosomal RNA from each group had a similar base composition, but although they had the same guanine–cytosine content of slightly over 61%, young microsomes and ribosomes contained significantly more guanine and less cytosine than adult. The difference in guanine–cytosine content between nuclear and ribosomal RNA could be explained if the nucleus contained about 20% of a messenger-type RNA with about 42% of guanine–cytosine. The difference in base ratio between microsomes and ribosomes from young and adult animals suggests that they consist of different populations, and therefore that a specific fraction is lost in the declining adult population.

It might be suggested that the lost fraction is that concerned with the additional protein requirements of the dividing cell, the fraction remaining in the adult cell being principally concerned with the synthesis of protein for cell maintenance.

I am grateful to Professor H. McIlwain for his interest, to Mr L. Lim for helpful discussions and to Mr H. Russell for skilled technical assistance. Financial support was kindly given by the Bethlem–Maudsley Hospital Research Fund.

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